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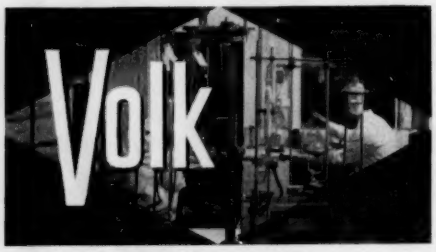

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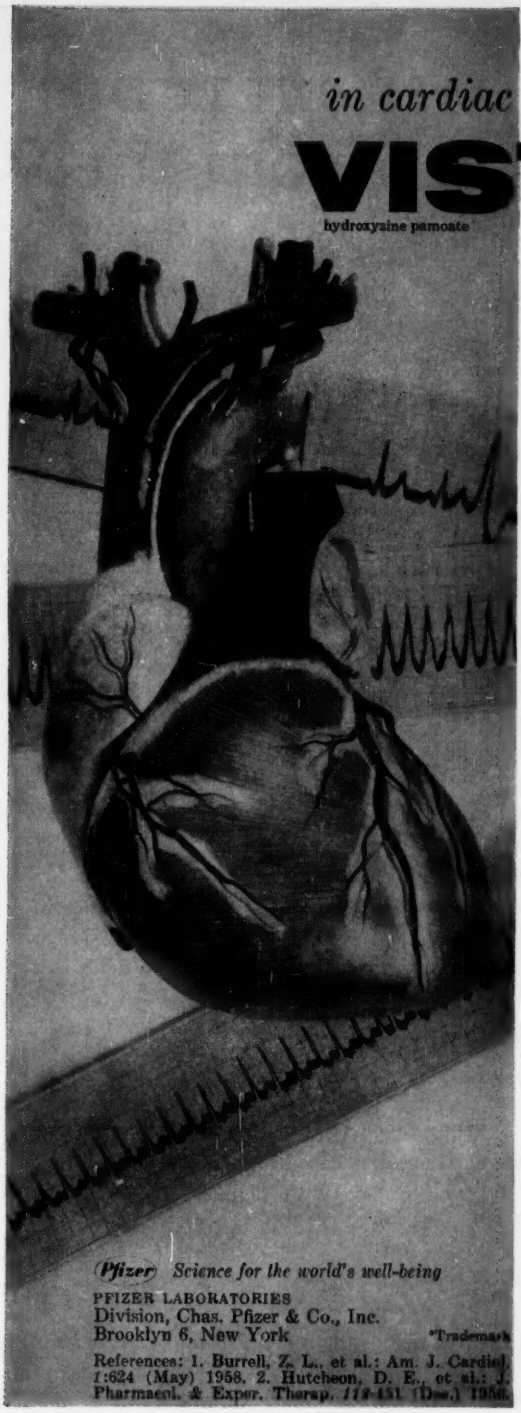

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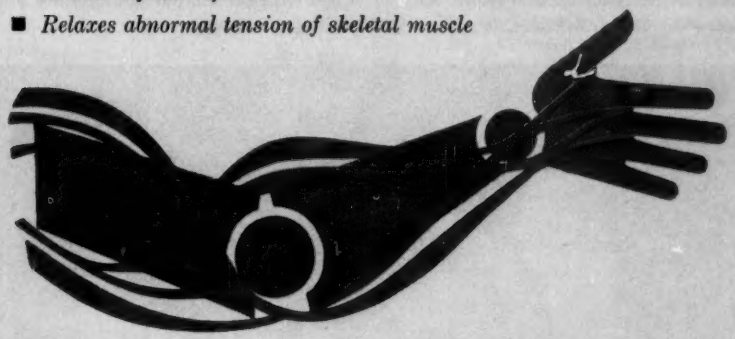

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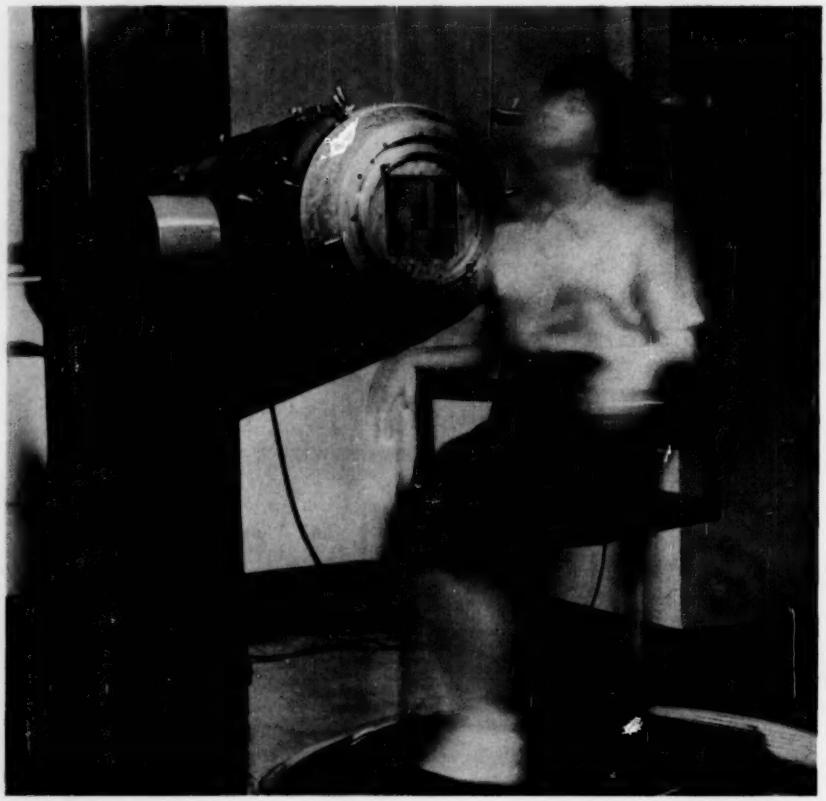

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AMMONIA AS A SOURCE OF GASTRIC HYPOACIDITY IN PATIENTS WITH UREMIA *

By CHARLES S. LIEBER †‡ AND ANDRÉ LEFÈVRE

(From the Department of Internal Medicine, Brussels University, and the Fondation Reine Elisabeth, Brugmann Hospital, Brussels, Belgium)

(Submitted for publication March 3, 1959; accepted March 26, 1959)

Ammonia is produced when urea passes from the blood into gastric secretion, a reaction catalyzed by a urease, present in the gastric mucosa of man and several animals (1–14). Fitzgerald and Murphy (15) have suggested that the ammonia formed in this process may have an important protective function by neutralizing gastric acidity. This concept was based on the observation in man that administration of urea in amounts sufficient to raise temporarily the blood urea level by 12 mg. per cent was followed by a decrease of gastric acidity. Burke and Page (16) were unable to confirm these findings, and Kornberg, Davies and Wood (17–19) presented evidence which suggested that, at least in the cat, the urease found in the stomach is of bacterial origin and does not influence gastric acid secretion.

This paper compares gastric ammonia and acid contents in normal and uremic subjects, the latter known to exhibit an elevated gastric ammonia concentration (20–22). Because of the influence of antibiotics on intestinal ammonia production (23–25), their effects on gastric ammonia and acid concentration were studied.

MATERIALS AND METHODS

Fifty hospitalized subjects were studied, 24 without evidence of renal dysfunction, 25 with uremia due to chronic renal disease, and one with azotemia due to obstructive uropathy. The blood urea concentration in the patients with uremia varied from 40 to 174 mg. nitrogen per cent with an average of 100.5 mg. N per cent. The patients were fasted for 16 hours and gastric juice collected by a Levine tube with the patients placed in the left lateral position, as proposed by Henning (26), to facilitate the quantitative recovery of gastric secretion (27). After a 45 minute period of stabilization, gastric juice was aspirated every 15 minutes and collected for examination. Basal secretion was studied using the four specimens collected during the first hour. At the end of this period, 0.005 mg. per Kg. histamine dihydrochloride was administered subcutaneously, and the effect of this drug on volume and composition of gastric secretion was determined on the four specimens collected during the second hour. This procedure was performed before and after administration of various antibiotics.[1] Twenty mg. per Kg. oxytetracycline was given orally for four to seven days to five nonuremic and six uremic subjects; 300 mg. of the same drug was injected intramuscularly daily for three to eight days to three nonuremics and three uremics. Five uremic and five nonuremic subjects received 20 mg. per Kg. chloramphenicol orally for four to seven days and six nonuremic and five uremic subjects were treated similarly with 20 mg. per Kg. erythromycin.

Venous blood urea was measured by the urease method (28), gastric juice was analyzed for chlorides (29) and free and total acid (dimethyl-aminoazobenzol and phenolphthalein as indicators). Ammonia and urea were measured (28) in neutralized gastric juice.

RESULTS

A. Comparison between gastric juice in uremic and nonuremic subjects

Increased blood urea was associated with high gastric urea and ammonia concentration. This was found both in the basal state and after administration of histamine. In addition, the free and the total acid concentrations were significantly lower ($p < 0.05$) in uremic patients as compared to nonuremic individuals (Table I).

B. Action of antibiotics on gastric ammonia and urea contents

Mean ammonia concentrations of basal and post-histamine gastric secretions were lowered significantly ($p < 0.05$) in the patients treated by oral oxytetracycline (Table II). In both non-

* Presented in abstract form at the World Congress of Gastroenterology, Washington, D. C., May, 1958.

† Supported by a grant from the Fonds National de la Recherche Scientifique Belge.

‡ Present address: Thorndike Memorial Laboratory, Boston City Hospital, Boston, Mass.

[1] The authors wish to thank the following companies for making these antibiotics available: Lepetit (chloramphenicol), Lilly (erythromycin) and Pfizer (oxytetracycline).

TABLE I

Comparison between gastric secretion of uremic and nonuremic subjects

		Free acid	Total acid	Chlorides	Gastric ammonia	Gastric urea	Blood urea
		mEq. HCl/L.	*mEq. HCl/L.*	*mEq./L.*	*mMole/L.*	*½ mMole/L.*	*½ mMole/L. (or mg. N %)*
24 subjects without uremia	Basal secretion	22.0	39.6	102.3	5.7	2.8	11.4 ± 0.6 (16.0 ± 0.8)
	S. D.	4.5	4.9	5.0	0.9	0.5	
	Posthistamine secretion	51.5	65.3	121.9	5.1	2.5	
	S. D.	7.4	7.6	5.5	0.9	0.5	
26 subjects with uremia	Basal secretion	8.1	28.2	119.1	37.4	15.9	71.8 ± 4.9 (100.5 ± 6.9)
	S. D.	4.1	4.3	5.1	5.2	3.7	
	Posthistamine secretion	32.8	55.4	137.7	31.8	17.4	
	S. D.	6.0	6.6	2.5	5.0	4.0	

uremic and uremic subjects the ammonia concentration was reduced by about 80 per cent of the pretreatment level. An increase of gastric juice urea concentration was associated with the change in ammonia. Both changes were of the same order of magnitude. In five of the six patients with uremia receiving oxytetracycline the drop of ammonia was accompanied by a rise of acidity, while in subjects without renal dysfunction no change in acidity was detectable. Similar results were obtained when the values were calculated on the basis of hourly gastric output (Figure 1). No change in

TABLE II

Gastric secretion before (I) and after (II) oral administration of oxytetracycline

	Case number	Free acid		Total acid		Chlorides		Ammonia		Urea		Blood urea	
		I	II	I	II	I	II	I	II	I	II	I	II
		mEq. HCl/L.		*mEq. HCl/L.*		*mEq./L.*		*mMole/L.*		*½ mMole/L.*		*½ mMole/L. (or mg. N %)*	
Nonuremic subjects													
Basal secretion	1	32	2	62	23	81	97	11.7	2.0	6.2	11.2	17.4 (24.4)	22.0 (30.8)
	2	84	112	102	120	140	134	4.0	1.8	0.6	3.6	10.5 (14.7)	12.8 (17.9)
	3	26	23	37	36	107	119	1.9	1.2	2.4	3.5	16.6 (23.3)	
	4	0	0	10	15	94	112	11.9	1.0	1.9	10.3	14.0 (19.6)	11.9 (16.7)
	5	0	4	5	14	72	81	2.7	0.8	3.5	6.6	8.2 (11.5)	11.4 (16.0)
	Mean	28.4	28.2	43.2	41.6	99	109	6.4	1.4	2.9	7.0	13.3 (18.6)	14.5 (20.3)
Posthistamine secretion	1	89	61	108	78	143	128	7.6	1.2	8.6	7.8		
	3	51	66	62	78	116	137	2.2	0.7	2.1	2.9		
	4	0	3	10	26	108	119	14.3	0.9	1.2	9.8		
	5	31	34	43	44	100	126	2.6	0.8	2.4	4.8		
	Mean	43	41	56	57	117	128	6.7	0.9	3.6	6.3		
Uremic subjects													
Basal secretion	6	0	26	24	47	127	119	79.0	12.0	0.6	70.0	110 (154)	124 (174)
	7							74.0	3.4	0	48.6	103 (144)	108 (151)
	9	0	0	10	13	98	96	27.6	14.0	5.2	45.6	52 (73)	82 (115)
	10	0	17	14	34	128	119	61.0	25.6	2.7	15.1	75 (105)	73 (102)
	11	0	10	13	20	122		32.9	2.0	0.3	22.4	54 (76)	33 (46)
	Mean	0	13	15	29	119	111	54.9	11.4	1.8	40.3	78.8 (110.3)	84 (117.6)
Posthistamine secretion	6	0.6	44	42	74	136	136	71.0	10.0	0.6	68.0		
	7	67	129	97	138	156	145	65.0	3.0	0.5	39.4		
	8	81	109	98	128	134	145	3.9	0.7	23.2	35.1	52 (73)	73 (102)
	9	51	87	73	108	139	148	34.7	14.4	1.2	27.1		
	10	45	37	64	60	136	134	42.0	24.8	0.5	13.1		
	11	0	7	11	13	133	84	30.5	1.9	1.5	23.5		
	Mean	41	69	64	87	139	132	41.2	9.1	4.6	34.3		

chloride content or volume of gastric juice was observed. Intramuscular administration of oxytetracycline was less effective than when given by mouth, both in reducing gastric ammonia and increasing acidity.

Neither gastric ammonia, acid, nor chloride was significantly influenced by chloramphenicol ($p > 0.05$) (Table III). Individual responses to erythromycin varied (Table IV); in some subjects gastric ammonia fell considerably, while in others there was no change. The differences were not found to be statistically significant ($p > 0.05$).

The observed antibiotic effect on gastric secretion was reversible. In four patients with uremia whose gastric ammonia had been reduced by antibiotics, it rose again after cessation of the treatment.

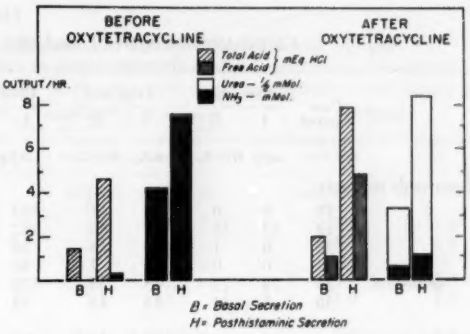

FIG. 1. GASTRIC SECRETION BEFORE AND AFTER SEVEN DAYS OF ORAL OXYTETRACYCLINE TREATMENT (20 MG. PER KG. DAILY)

Uremic patient (blood urea, 154 to 174 mg. N per cent).

C. Relationship between changes in ammonia and acid concentrations in gastric secretion

The relationship between spontaneous changes in gastric ammonia and acid concentration was studied in the patient in whom azotemia due to obstructive uropathy was relieved by cystostomy. The gastric ammonia content was lowered proportionally and gastric acidity rose to a similar extent with the fall of blood urea (Figure 2). Ad-

TABLE III

Gastric secretion before (I) and after (II) oral administration of chloramphenicol

	Case number	Free acid		Total acid		Chlorides		Ammonia		Urea		Blood urea	
		I	II	I	II	I	II	I	II	I	II	I	II
		mEq. HCl/L.		*mEq. HCl/L.*		*mEq./L.*		*mMole/L.*		*½ mMole/L.*		*½ mMole/L. (or mg. N %)*	
Nonuremic subjects													
Basal secretion	12	54	26	72	41	130	124	11.1	5.1	0.4	2.3	13.9 (19.5)	12.7 (17.8)
	13	21	16	46	22	126	124	11.5	5.4	1.5	3.0	15.4 (21.6)	10.0 (14.0)
	14	36	60	47	68	105	123	3.5	3.1	1.8	0.4	10.0 (14.0)	6.0 (8.4)
	15	0	7	6	22	55	107	2.6	3.0	3.9	3.3	14.0 (19.6)	10.9 (15.3)
	16	0	0	11	4	72	146	4.8	0.5	8.9	9.3	15.9 (22.3)	14.5 (20.3)
	Mean	20	22	36	31	97	125	6.7	3.4	3.3	3.7	13.8 (19.3)	10.8 (15.1)
Posthistamine secretion	12	92	99	110	111	155	160	7.1	3.8	1.2	0.9		
	13	63	44	78	73	143	134	9.4	6.3	0.7	2.1		
	14	61	75	70	91	128	143	3.1	2.5	1.7	0.4		
	15	48	53	58	73	128	135	5.1	2.5	3.0	2.5		
	16	0	0	12	10	60	138	3.6	1.8	9.1	10.2		
	Mean	53	54	66	72	123	142	5.7	3.4	3.1	3.2		
Uremic subjects													
Basal secretion	17	0	18	14	42	128	138	61.0	52.1	2.7	0.2	75.0 (105.0)	62.5 (87.5)
	18	0	0	4	12	140	134	47.0	48.0	18.0	20.0	74.0 (104.0)	81.0 (113.4)
	19	6	35	22	56	107	158	8.6	12.0	20.5	22.6	45.5 (63.7)	50.0 (70.0)
	38	0	20	12	40	122	105	34.7	3.4	2.2	31.2	48.5 (67.9)	50.1 (70.1)
	39	0	3	7	23	113	130	23.3	14.2	1.8	4.4	30.0 (42.0)	28.6 (40.0)
	Mean	1.2	15.2	11.8	34.6	122	133	34.9	25.9	9.0	15.7	54.6 (76.4)	52.4 (73.4)
Posthistamine secretion	17	45	57	64	78	136	154	42.0	45.0	0.5	0.3		
	18	0	0	8	14	150	146	48.0	46.0	25.0	28.0		
	19	46	74	66	95	141	160	10.9	8.5	13.2	15.9		
	38	0	25	11	38	122	106	34.6	3.8	3.5	35.8		
	Mean	22.8	39.0	37.3	56.3	137	142	33.9	25.8	10.6	20.0		

TABLE IV

Gastric secretion before (I) and after (II) oral administration of erythromycin

	Case number	Free acid		Total acid		Chlorides		Ammonia		Gastric urea		Blood urea	
		I	II	I	II	I	II	I	II	I	II	I	II
		mEq. HCl/L.		*mEq. HCl/L.*		*mEq./L.*		*mMole/L.*		*½ mMole/L.*		*½ mMole/L. (or mg. N %)*	
Nonuremic subjects													
Basal secretion	21	0	0	16	17	102	138	6.7	2.3	1.1	5.2	10.6 (14.8)	10.0 (14.0)
	22	32	31	44	46	102	116	1.3	1.7	4.4	7.1	8.3 (11.6)	9.9 (13.9)
	23	0	1	7	13	98	89	8.5	3.5	0.8	1.4	11.5 (16.1)	9.0 (12.6)
	24	0	0	5	7	80	90	3.5	2.1	4.9	5.8	9.3 (13.0)	10.0 (14.0)
	25	28	22	45	35	120	77	18.3	6.2	0.2	0.5	15.2 (21.3)	12.6 (17.6)
	36	5	34	15	45	94	104	4.3	7.3	1.8	1.1	7.2 (10.1)	9.2 (12.9)
	Mean	10.8	14.5	22.0	27.2	99	102	7.1	3.9	2.2	3.5	10.4 (14.6)	10.1 (14.1)
Posthistamine secretion	21	0	30	27	57	108	136	5.3	0.8	1.0	3.4		
	22	67	86	74	95	126	146	0.9	0.9	3.5	4.5		
	23	27	50	43	67	109	119	8.8	3.5	0.1	1.4		
	24	0	0	4	10	73	90	3.7	1.7	4.3	5.8		
	25	48	55	61	77	122	121	16.2	6.0	0.1	0.6		
	36	53	87	65	96	115	125	5.0	6.8	0.8	0.3		
	Mean	32.5	51.3	45.7	67	109	123	6.7	3.3	1.6	2.7		
Uremic subjects													
Basal secretion	26	2	1	28	17	145	74	64.2	12.5	0	13.7	70 (98)	29 (41)
	27	0	0	15	33	128	107	32.5	0.5	1.5	29.5	69 (97)	40 (56)
	28	10	58	32	98	117	151	54.7	7.9	23.5	76.1	96 (134)	113 (158)
	29	0	0	5	3			7.8	6.3	24.4	14.3	39 (55)	30 (42)
	49	7	6	33	22	138	107	15.2	8.6	20.6	20.5	46 (64)	46 (64)
	Mean	3.8	13	23	35	132	110	34.9	7.2	14.0	30.8	64.0 (89.6)	51.5 (72.1)
Posthistamine secretion	26	40	54	69	70	142	111	46.6	9.4	0	10.3		
	27	0	55	32	84	134	153	23.0	1.4	17.7	22.8		
	28	54	68	81	102	136	152	32.0	8.9	46.0	83.5		
	29	0	0	10	8			8.2	6.0	12.4	12.6		
	49	38	46	58	66	150	141	15.5	10.9	14.4	13.2		
	Mean	26.4	44.6	50.0	66.0	141	139	25.1	7.3	18.1	28.5		

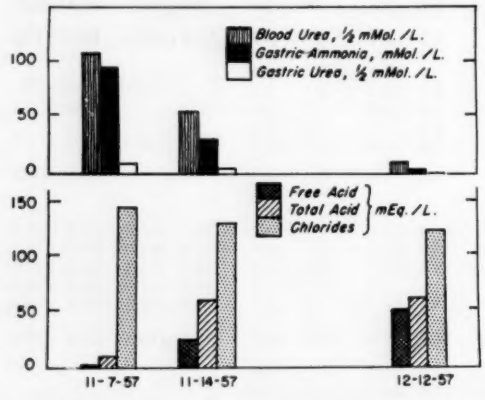

FIG. 2. EFFECTS OF A DECREASE IN BLOOD UREA ON GASTRIC AMMONIA AND ACIDITY (POSTHISTAMINE SECRETION)

ministration of antibiotics resulted in a significant drop of gastric ammonia ($p < 0.01$) and a significant increase of gastric acidity ($p < 0.05$) in eight patients with uremia due to a chronic glomerulonephritis in whom no change in blood urea occurred during the period of investigation (Table V, Group A). In a similar group (Table V, Group B) of nine patients with uremia, where no marked reduction of gastric ammonia occurred, no change of acidity was observed.

DISCUSSION

Several authors (14, 15, 22, 30) have shown that the concentration of ammonia in gastric juice is roughly proportional to the blood urea level; this has been confirmed by our results.

Anemia, lowered blood carbon dioxide content,

TABLE V

Seventeen patients with stable uremia: Gastric secretion before and after antibiotic treatment with (A) and without (B) significant reduction in gastric ammonia

		Before antibiotic treatment		After antibiotic treatment		Difference	
		Gastric ammonia	Free acid	Gastric ammonia	Free acid	Gastric ammonia	Free acid
		mMole/L.	*mEq. HCl/L.*	*mMole/L.*	*mEq. HCl/L.*	*mMole/L.*	*mEq. HCl/L.*
Group A (8 uremic patients)	Basal secretion	49.8	1.4	7.6	18.7	−42.2	+17.3
	S. D.	6.6	1.4	3.4	7.5	5.2	6.3
	Posthistamine secretion	41.0	21.6	5.6	52.2	−35.4	+30.6
	S. D.	6.1	10.6	1.7	15.0	6.0	7.7
Group B (9 uremic patients)	Basal secretion	26.7	12.8	23.1	12.7	− 3.6	− 0.1
	S. D.	6.9	9.8	6.7	6.7	2.1	12.3
	Posthistamine secretion	24.0	46.2	19.2	47.4	− 4.8	+ 1.2
	S. D.	5.9	10.6	5.7	11.6	2.0	7.0

disturbances of water and electrolyte balance (31) and gastritis (32) have been suggested previously as causes for gastric hypoacidity in uremia. Although it is possible that these factors may contribute to the encountered low gastric acidity, no experimental proof that they are responsible has been presented.

The hypothesis that gastric ammonia reduces acidity (15) seemed more plausible but has not been supported previously by experimental evidence in uremic patients.

The demonstration that reduction of gastric ammonia after antibiotic treatment increased acidity of patients with uremia and *in vitro* addition of ammonia decreased acidity (33) clearly relates hypoacidity to ammonia.

In uremic patients, reduction of gastric ammonia causes gastric acidity to rise to a level similar to that of nonuremic subjects. In the basal state, where gastric acid secretion is normally low, the decrease in ammonia may be greater than the increase of acidity. After histamine stimulation, the ammonia decrease parallels the increased acidity.

Administered antibiotics did not influence gastric acidity in the absence of marked changes of ammonia. This was seen in both control subjects and uremic patients in whom antibiotics had little or no effect on gastric ammonia. The encountered differences in ability of oxytetracycline, erythromycin and chloramphenicol to influence gastric ammonia are unexplained; they may be related to the different antibiotic spectra or pharmacodynamic effects. Since bacteria reside in the stomach under physiological conditions (34, 35) and urease activity of gastrointestinal organism is reduced by antibiotics (36–39), it seems probable that the ammonia-reducing effect of oxytetracycline and erythromycin is related to their antibiotic properties rather than to a pharmacodynamic effect.

After use of oxytetracycline, an increased incidence of blood in gastric juice was noted and may be attributed to the occurrence of gastric ulcerations after such treatment (40). Theoretically, return of normal acidity coupled with the effects of retained urinary excretory products on mucous membranes may predispose to ulcerations and gastroduodenal bleeding (41).

Effects of therapy on gastric ammonia and acidity may also be of practical importance in patients with liver disease and azotemia who exhibit an elevated gastric ammonia. Reduction of gastric ammonia in patients with cirrhosis can be achieved with oxytetracycline (42) or neomycin (43).

SUMMARY

Twenty-six patients with uremia had high gastric ammonia and low free and total acid concentrations, compared with 24 control subjects without evidence of renal dysfunction.

Spontaneous and induced reduction of gastric ammonia in patients with uremia was accompanied by an equivalent increase in gastric acidity, indicating the contribution of ammonia to hypoacidity in uremia.

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EVIDENCE THAT A HUMORAL AGENT STIMULATES THE ADRENAL CORTEX TO SECRETE ALDOSTERONE IN EXPERIMENTAL SECONDARY HYPER-ALDOSTERONISM *

By NICHOLAS A. YANKOPOULOS, JAMES O. DAVIS, BERNARD KLIMAN and RALPH E. PETERSON with the surgical assistance of ALFRED CASPER

(From the Section on Experimental Cardiovascular Disease, Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute and the National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.)

(Submitted for publication December 16, 1958; accepted April 2, 1959)

The mechanisms which effect alterations in the secretion of aldosterone by the adrenal cortex may be nervous, humoral or both. The present study was undertaken to examine the possibility that a humoral agent stimulates the adrenal cortex to secrete aldosterone in dogs with experimental secondary hyperaldosteronism produced by constriction of the thoracic inferior vena cava. These animals secrete large amounts of aldosterone (1) and show almost complete retention of Na (2). To test the hypothesis of a humoral mechanism, the effect on aldosterone secretion was studied in normal recipient animals or in the isolated adrenals of normal dogs during cross-circulation of blood from dogs with constriction of the thoracic inferior vena cava. Corticosterone secretion was also measured to evaluate the possible role of adrenocorticotropic hormone (ACTH) in the aldosterone regulatory system.

METHODS

The basic plan of the experiments consisted of cross-circulation of blood from a donor into a recipient animal or into the isolated adrenal glands of a recipient animal. Secondary hyperaldosteronism was produced in the donor animals by either acute or chronic constriction of the thoracic inferior vena cava. Acute caval constriction was performed on normal dogs. In the chronic donor dogs, ascites had been present for one to three weeks, and Na excretion on the day before the experiment was less than 4 mEq. per day; Na and K intakes were 60 and 18 mEq. per day, respectively. Also, aldosterone secretion from the right adrenal gland was measured in a group of eight dogs with chronic thoracic inferior vena cava constriction to determine the magnitude and consistency of hypersecretion. All animals were mongrel dogs which weighed 15 to 20 Kg. Nembutal® anesthesia was used. Each dog received 15,000 I.U. of heparin before collection of adrenal vein blood and before cross-circulation.

Two types of experiments were performed. In the first group of experiments (three pairs of dogs), cross-circulation was achieved through the femoral vessels by polyethylene tubing (i.d., 3.5 mm.). Exchange of blood was regulated by screw clamps so that arterial and venous pressures remained similar to the values recorded before cross-circulation. Adrenal vein blood was collected from both animals from the right adrenal gland by cannulation of the right adrenolumbar vein as described previously (1). In the first pair of dogs, cross-circulation was performed between two normal animals. After control determinations in both animals, the thoracic inferior vena cava was constricted in one of the dogs which became the donor. In the two other experiments of this group, dogs with chronic thoracic caval constriction were the donor animals. Under these circumstances, control observations were made in both the chronic donor and the normal recipient before cross-circulation was established.

In the second group of experiments (15 pairs of dogs), blood was circulated from a donor animal with chronic thoracic caval constriction and ascites or from a normal dog into the isolated adrenals of a normal recipient animal (Figure 1). Cross-circulation of blood from normal dogs provided a control on the effect of cross-circulation *per se*. The recipient's adrenals were isolated *in situ* with segments of aorta and inferior vena cava by the procedure of Hilton and co-workers (3). Care was taken to include in the arterial circulation to the lower pole of the right adrenal gland a small artery arising from the aorta. Polyethylene tubing was used for all connections. During the control and recovery periods, blood was circulated from the recipient's carotid artery (autoperfusion) into the isolated aorta, through the adrenal glands, into the inferior vena cava and, subsequently, into the systemic circulation of the recipient by way of the external jugular vein. During the period of cross-circulation, blood perfused the isolated adrenals of the recipient from the femoral artery of the donor and returned to the donor's femoral vein. An infusion of norepinephrine (100 μg. per ml.) was given in some instances (Tables I and II) during the latter part of the experiment to support arterial pressure and adrenal blood flow.

Collections of 10 ml. of adrenal vein blood and 10 ml. of peripheral blood were made at approximately one-half

* A preliminary report was given at the Laurentian Hormone Conference, September, 1958.

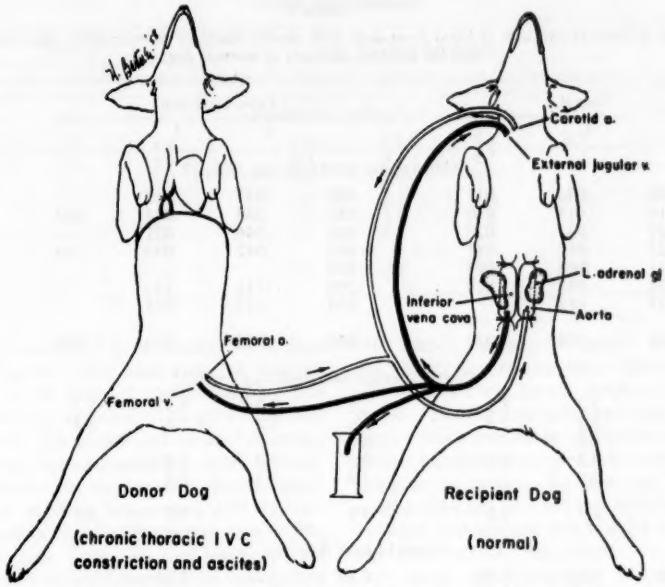

FIG. 1. CROSS-CIRCULATION OF BLOOD FROM A DOG WITH CHRONIC THORACIC INFERIOR VENA CAVA (IVC) CONSTRICTION INTO THE ISOLATED ADRENALS OF A NORMAL RECIPIENT ANIMAL

hour intervals; blood was replaced immediately. Adrenal blood flow was measured by collection of blood into a graduated tube; the hematocrit was determined and adrenal plasma flow was calculated. The concentrations of aldosterone and corticosterone in adrenal vein plasma were determined and the rates of secretion calculated. The concentrations of plasma Na and plasma K were determined during cross-circulation of blood from dogs with hyperaldosteronism. Arterial and venous pressures were measured continuously with Statham strain gages and a Sanborn recording system. During cross-circulation via the femoral vessels, aortic and inferior vena caval pressures were determined in both donor and recipient animals. During perfusion of the isolated adrenals, pressures were recorded from the isolated segments of the aorta and inferior vena cava associated with the adrenals. The height of the animal table of the recipient dog was adjusted to maintain a constant venous pressure in the inferior vena cava or in the isolated segment of the inferior vena cava.

Procedure for the double isotope derivative assay for aldosterone and corticosterone. Aldosterone and corticosterone were measured in 3 ml. of adrenal vein plasma by the double isotope derivative procedure of Kliman, Peterson, Davis and Yankopoulos (4, 5). Each 3 ml. sample of plasma was extracted with 22 ml. of purified dichloromethane. The dichloromethane extract was washed successively with 2 ml. of 0.05 N NaOH and with 2 ml. of 0.1 N acetic acid. The dichloromethane extract was dried at 37° C. with a stream of nitrogen. The dried extract was transferred to a 6.5 ml. conical glass-stoppered tube with 2 to 3 ml. of absolute ethanol and two rinses of 2 ml. each of dichloromethane; the sample was dried with a stream of nitrogen. Each sample was acetylated with 0.03 ml. of tritium-labeled acetic anhydride (15 per cent acetic anhydride in dry benzene) in the presence of 0.025 ml. of anhydrous pyridine. The specific activity of the acetic anhydride was approximately 100 mc. per mMole. Each sample was incubated for 60 to 70 hours at 37° C.; the acetylation tube was rotated on one occasion during incubation to insure complete acetylation. Aldosterone diacetate-H^3, corticosterone monoacetate-H^3 and the labeled acetates of other adrenal steroids were formed. About 2,000 cpm of aldosterone diacetate-C^{14} in 0.1 ml. of ethanol and 5,000 cpm of corticosterone monoacetate-C^{14} in 0.1 ml. of ethanol were added to each sample to correct for losses of aldosterone and corticosterone during all subsequent chromatographies. The exact number of cpm for each steroid in 0.1 ml. of ethanol was determined when the labeled steroids from the plasma samples were counted. Distilled water (0.8 ml.) was added to each tube, extraction was carried out with about 5 ml. of dichloromethane and the dichloromethane extract was washed once with 1 ml. of distilled water. The dichloromethane was dried under a stream of air at 37°. Adrenosterone (0.02 ml.) and corticosterone monoacetate (0.02 ml.) in concentrations of 1 mg. per ml. in absolute ethanol were added to each sample to provide a visible marker in ultraviolet light for aldosterone and enough corticosterone monoacetate for it to be visible.

TABLE I

Effects of cross-circulation of blood from dogs with chronic thoracic inferior vena cava constriction into the isolated adrenals of normal dogs

Dog no.	Control			Cross-circulation				Recovery	
	1	2	3	1	2	3	4	1	2
				Aldosterone secretion (μg./min.)*					
4	.006	.015	.017	.025	.037	.019			
5	.019	.015	.010	.051	.038	.014	.045	.019	.011
6	.029	.047	.026	.064	.046	.021		.018	.016
7	.022	.012	.011	.034	.042	.039	.035	.011	.022
8	.011	.005	.005	.037					
9	.054	.048	.049	.100	.141	.146		.063	.053
10	.027	.018		.039	.047	.058		.043	.024
Mean	.024	.023	.020	.050	.059	.050	.040	.031	.025
				Corticosterone secretion (μg./min.)*					
5		2.2	1.9	5.5	3.0	1.7	3.3	1.5	1.0
7	2.2	2.2	2.3	3.1	3.4	2.6	2.4	2.0	2.6
8	2.7	3.1		3.0					
9	3.4	3.2	3.2	4.6	6.0	5.6		2.2	2.0
10	2.0	2.0		2.0	2.3	2.1		1.9	1.1
Mean	2.6	2.5	2.5	3.6	3.7	3.0	2.9	1.9	1.7
				Adrenal blood flow (ml./min.)*					
4	7.40	6.67	5.50	6.80	3.22	1.84			
5	12.50	11.33	10.17	16.24	7.50	4.03	9.60	10.52	7.69
6	11.30	8.44	7.70	8.11	4.80	3.33		8.11	8.00
7	13.33	7.41	7.06	10.00	8.11	5.36	8.83	7.06	8.00
8	10.75	8.90	7.10	9.50					
9	7.50	6.17	6.13	12.00	11.76	13.60		4.59	3.57
10	7.50	6.54	5.81	2.93	2.89	3.01		3.54	2.62
Mean	10.04	7.92	7.07	9.37	6.38	5.19	9.21	6.76	5.98
				Mean arterial pressure (mm. Hg)*					
4	125	125	125	120	70	70			
5	140	140	140	125	95	90	130†	200	170
6	120	135	130	95	100†	100†		120	130
7	135	145	135	95	95	80	140†	160	135
8	115	120	100	155†					
9	114	103	92	100	96	87		98	90
10	122	130	127	92	86	96		114	112
Mean	124	128	121	112	90	87	135	138	127
				Venous pressure (mm. water)*					
5	55	60	60	40	30	25	20	145	120
6	35	30	35	30	45	50		70	55
7	65	65	55	55	50	70	80	80	60
8	90	75	70	50					
9	45	50	55	45	30	30		45	38
10	65	55	50	35	20	15		40	45
Mean	59	56	54	43	35	38	50	76	63
				Plasma Na (mEq./L.)					
4	152	149	152	145	149	149	165		
5	142	144	144	143	145	144	146	149	152
6	149	147	147	149	154	151		152	151
7	150	148	145	140	149	149	146	146	150
9	136	145	146	140	141	146		146	143
10	145	150	146	136	136	139		144	147
Mean	146	147	147	142	146	146	152	147	149

* Steroid secretion, adrenal blood flow and arterial and venous pressures from isolated adrenals and associated vessels.
† Norepinephrine infusion given to maintain arterial pressure and adrenal blood flow.

TABLE I—*Continued*

Dog no.	Control			Cross-circulation				Recovery	
	1	2	3	1	2	3	4	1	2
				Plasma K (mEq./L.)					
4	3.4	3.8	3.6	3.4	4.0	3.7	3.7		
5	4.1	3.6	3.9	4.5	3.7	4.2	3.4	2.8	
6	3.9	3.2	3.0	3.9	4.0	4.7		2.5	
7	3.7	3.3	3.3	3.4	4.1	5.0	4.5	5.5	5.0
9	3.2	3.0	2.8	2.9	2.6	4.1		3.7	3.9
10	4.3	4.0	3.8	4.3	4.5	4.7		4.0	4.2
Mean	3.8	3.5	3.4	3.7	3.8	4.4	3.9	3.7	4.4

Each sample was transferred to Whatman No. 1 filter paper (7.5 × 22.5 inches) with four drops of absolute ethanol and eight to 10 drops of dichloromethane and chromatographed in a cyclohexane (100 parts), benzene (40 parts), methanol (100 parts) and water (20 parts) system by descending chromatography. After a minimal period of 45 minutes for equilibration, the chromatogram was developed at room temperature (75° F.) for 16 to 18 hours by adding 90 ml. of the mobile phase. The paper was scanned with ultraviolet light, each spot of corticosterone monoacetate and adrenosterone was marked and areas of paper containing corticosterone monoacetate and aldosterone diacetate were cut out. The relation of adrenosterone to aldosterone diacetate had been previously ascertained by locating the position of aldosterone diacetate-C^{14} with a strip counter and the visible spot of adrenosterone by ultraviolet light. This relationship was checked periodically. The samples of corticosterone monoacetate and aldosterone diacetate were eluted into separate tubes with 10 ml. of absolute methanol. Both samples were dried.

For aldosterone, the second chromatography was carried out with a cyclohexane (100 parts), dioxane (100 parts), methanol (50 parts) and water (25 parts) system. The reference marker for aldosterone diacetate in this chromatographic system was 11-keto, 17αhydroxyprogesterone (0.02 ml. of a 1 mg. per ml. solution); the exact relation of the mobility of this steroid and aldosterone had been determined previously. The chromatogram was developed for 14 to 16 hours, the aldosterone diacetate was eluted with methanol and the sample was dried under air. To alter the mobility of the steroid and to insure better purification, each sample was treated with 0.1 ml. of 0.5 per cent chromic trioxide in glacial acetic acid. The tube was rotated to allow the chromate solution to wet the residue and allowed to stand for 10 minutes. The reaction was stopped with 2 ml. of 20 per cent absolute ethanol in water. Approximately 10 ml. of dichloromethane was added and each tube was shaken vigorously; the aqueous layer was aspirated and discarded. Each sample was washed twice with 1 ml. of distilled water and the dichloromethane was dried under an air stream.

The third chromatography for the oxidation product of aldosterone diacetate was performed in a cyclohexane (100 parts), benzene (50 parts), methanol (100 parts) and water (25 parts) system. The reference marker was 0.02 ml. of a 1 mg. per ml. solution of 11αhydroxyprogesterone. The chromatogram was developed for 24 to 26 hours. Each sample of the oxidation product of aldosterone diacetate was eluted into a counting vial and dried under an air stream. To each vial, 5 ml. of a phosphor solution (0.4 per cent solution of 2,5-diphenyloxazole and 0.004 per cent solution of 1,4, di-2-5 phenyloxazole in reagent grade toluene) was added.

A second chromatography for corticosterone monoacetate was performed with a cyclohexane (100 parts), dioxane (100 parts), methanol (50 parts) and water (25 parts) system. The chromatogram was developed for 12 to 15 hours. The corticosterone was eluted into counting vials, dried and the phosphor solution added.

The samples of aldosterone and of corticosterone were counted to determine the tritium and carbon-14 radioactivity in a liquid scintillation spectrometer (Packard Tri-Carb Scintillation Spectrometer). Each sample was counted at a voltage of 890 (discriminator setting of 10 to 50) to determine the radioactivity of carbon-14 and at 1,270 volts (discriminator setting of 10-∞) to obtain the combined activity of carbon-14 and tritium. The number of tritium counts at 1,270 volts was obtained by subtracting the contribution of carbon-14 at this voltage from the total counts; the counts of carbon-14 at the high voltage were calculated from the ratio of pure carbon-14 counts at 1,270 volts to carbon-14 counts at 890 volts. The quantity of aldosterone or corticosterone in a sample was calculated from the yield of tritium radioactivity, the amount of carbon-14 lost during chromatography and the specific activity of the tritium-labeled acetic anhydride.

The specific activity of the tritium-labeled acetic anhydride was determined by acetylating hydrocortisone and determining the specific activity of the acetylated steroid. Dry hydrocortisone (0.5 mg.) was acetylated for 20 hours at room temperature with 0.02 ml. of tritium-labeled acetic anhydride in the presence of 0.02 ml. of anhydrous pyridine. After acetylation, 1 ml. of 20 per cent ethanol in water was added to the reaction mixture. The hydrocortisone acetate was extracted from the ethanol solution with five volumes of dichloromethane. The dichloromethane was washed with 1 ml. of distilled water and dried under an air stream. The sample of hydrocor-

TABLE II

Effects of cross-circulation of blood from normal dogs into the isolated adrenals of normal animals

Dog no.	Control			Cross-circulation			Recovery	
	1	2	3	1	2	3	1	2
				Aldosterone secretion (μg./min.)*				
11	.032	.034	.036	.047	.024		.043	.024
12	.062	.062	.056	.034	.023	.033†	.065	.063
13	.037	.045	.051	.022	.017	.025	.043	.070
14	.043	.010	.019	.016				
15	.038	.034	.032	.047	.047	.053	.032	.047
16	.073	.060	.053	.053	.064		.078	.118
17	.044	.014	.012	.009	.011	.019	.024	.051
18	.012	.005	.005	.019	.022	.032	.010	.009
Mean	.043	.033	.033	.031	.030	.032	.042	.055
				Corticosterone secretion (μg./min.)*				
11	3.5	4.2	3.6	3.1	2.9		3.7	1.9
12	3.8	3.8	3.3	4.7	4.5	4.4	3.2	3.2
13	3.9	3.4	3.6	4.3	3.3	2.6	2.4	3.2
14	3.3	2.4	2.5	4.5				
15	4.4	4.0	4.2	4.4	3.3	3.6	1.6	1.9
16	4.1	3.4	3.6	4.0	4.1		3.3	4.3
17	2.6	1.7	1.6	1.9	2.5	1.7	1.1	1.8
18	1.4	1.0	0.8	3.4	2.5	2.8	0.9	0.9
Mean	3.4	3.0	2.9	3.8	3.3	3.0	2.3	2.5
				Adrenal blood flow (ml./min.)*				
11	6.10	4.77	4.59	3.31	3.48		3.16	2.32
12	9.50	5.33	4.93	8.33	6.37	10.30	4.35	3.85
13	5.35	3.48	3.00	5.35	3.64	3.33	3.17	4.05
14	8.60	4.80	4.30	15.00				
15	3.92	3.75	3.02	5.82	3.17	2.92	1.10	2.40
16	11.30	7.80	9.10	11.10	7.80		10.00	9.80
17	12.80	4.92	4.00	5.22	3.83	4.42	2.22	3.33
18	10.20	7.14	5.72	7.57	6.26	6.89	3.94	3.45
Mean	8.47	5.24	4.83	7.71	4.93	5.57	3.99	4.17
				Mean arterial pressure (mm. Hg)*				
11	113	110	100	80	92‡		140‡	122‡
12	135	132	122	118	110	108	140	135
13	122	112	100	104	80	80	105‡	132‡
14	110	105	100	110				
15	80	96	101	125	115	103	77	140‡
16	125	125	116	116	94		134	134
17	110	127	125	112	81	95	106‡	114‡
18	110	123	116	110	92	102	118	116
Mean	113	116	110	109	95	98	117	128
				Venous pressure (mm. water)*				
11	0	5	20	−10	−10		0	50
12	30	20	20	15	20	10	25	20
13	30	20	20	20	20	0	0	10
14	−15	−20	−25	25				
15	30	35	30	40	40	40	0	110
16	45	35	25	25	20		45	40
17	20	20	25	0	−15	−10	90	100
18	80	65	55	20	30	30	70	75
Mean	28	23	21	17	15	14	33	58

* Steroid secretion, adrenal blood flow and arterial and venous pressures from isolated adrenals and associated vessels.

† The thoracic inferior vena cava was constricted for one hour and studies conducted between the experimental and recovery periods in this experiment (see Figure 4).

‡ Norepinephrine infusion given to maintain arterial pressure and adrenal blood flow.

tisone acetate was chromatographed on Whatman No. 1 paper for 15 to 18 hours in a carbon tetrachloride (100 parts), methanol (100 parts) and water (25 parts) system. The hydrocortisone acetate was eluted with ethanol and rechromatographed for 20 to 25 hours on alcohol washed paper in the second solvent system used for aldosterone. The hydrocortisone acetate was eluted with redistilled absolute ethanol and diluted to 25 ml. The concentration of hydrocortisone acetate in this eluate was measured by three methods: *1*) absorption in the ultraviolet in a Beckman DU spectrophotometer at 242 mμ ($\epsilon = 15{,}850$); *2*) phenylhydrazine reaction in sulfuric acid-ethanol (Porter-Silber) (6); and *3*) fluorometric assay with sulfuric acid-ethanol. The specific activity was determined by counting an aliquot of the eluate in the liquid scintillation spectrometer. The specific activity was expressed in terms of cpm per μg. at the 1,270 voltage setting.

The aldosterone diacetate-C^{14} was prepared from 1.0 mg. of aldosterone. The aldosterone was acetylated for 44 hours at 37° C. with acetic anhydride-1-C^{14} (5 mc. per mMole) in dry benzene (15 per cent) in the presence of anhydrous pyridine. The aldosterone diacetic-C^{14} was partitioned between aqueous alcohol and dichloromethane as described previously and chromatographed on Whatman No. 1 paper in the first and second systems described previously for aldosterone. The steroid was eluted with redistilled ethanol and diluted to a volume such that each 0.1 ml. yielded approximately 2,000 cpm. The solution was stored at −20° C. where it remained stable for months.

The corticosterone monoacetate-C^{14} was prepared from 2 mg. of corticosterone. Corticosterone was acetylated for 44 hours at 37° C. with 0.02 ml. of acetic anhydride-1-C^{14} (5 mc. per mMole) in benzene (15 per cent) in the presence of anhydrous pyridine. The corticosterone monoacetate-C^{14} was partitioned between aqueous alcohol and dichloromethane and chromatographed on Whatman No. 1 paper for 18 hours in the solvent system used for the first chromatography of aldosterone and corticosterone. The steroid was eluted, dried with a stream of nitrogen and chromatographed for 15 hours on the second solvent system used for aldosterone. The corticosterone was eluted with redistilled absolute ethanol and diluted to yield approximately 5,000 cpm per 0.1 ml.

Specificity, reproducibility and recovery data for the double isotope derivative assay for aldosterone. Evidence for the specificity of this procedure for aldosterone was obtained by demonstrating the constancy of H^3/C^{14} ratios for each sample with repeated chromatography. Aldosterone was added to each of the eight samples of pooled adrenal vein plasma in amounts to produce four levels of concentration (Table III); assays were carried out at each level and on the same pool of adrenal vein plasma with no added aldosterone. The H^3/C^{14} ratio was determined after each of the three chromatographies by determining tritium and carbon counts from an aliquot of the eluate at the 1,270 voltage setting. The solvent systems and time for development of the chromatograms were the same as those described previously for aldosterone. The ratio of H^3/C^{14} was essentially the same after the second and third chromatographies (Table III). This finding indicates that no tritium-labeled impurities were present after the second chromatography. Nevertheless, a third chromatography has been used in all routine studies. Recovery data on the same plasma samples are presented in Table III.

A similar study of the specificity of this technique for corticosterone was made. The first two chromatographies were the same as those used for corticosterone in routine

TABLE III

Recovery, reproducibility and specificity data for aldosterone with the double isotope derivative assay procedure

Sample	Aldosterone recoveries				Purification during chromatography		
					H^3/C^{14} after each chromatography‡		
	Aldosterone added*	Determined aldosterone	Recovery of aldosterone added	% Recovery†	1st	2nd	3rd
	μg./ml. of plasma	*μg./ml.*	*μg./ml.*				
N*		.025			21.1	2.14	2.32
1 A	.012–.015	.039	.014	104	34.7	4.18	4.40
B	.012–.015	.041	.016	119	15.0	1.88	2.21
2 A	.024–.030	.047	.022	82	36.4	5.58	5.28
B	.024–.030	.052	.027	100	17.2	2.67	2.90
3 A§	.040–.050	.075	.050	111	18.0	3.75	4.03
B	.040–.050	.065	.040	89	16.8	3.36	3.47
4 A§	.080–.100	.105	.080	89	16.6	5.77	5.62
B	.080–.100	.111	.086	96	15.3	6.13	5.96

* Three ml. of the same pool of adrenal vein plasma from the isolated adrenals of a normal dog were used for each sample. No aldosterone was added to Sample N. The concentration of the aldosterone added was determined by measuring a stock solution in the ultraviolet at 242 mμ ($\epsilon = 15{,}850$). The higher values given represent added aldosterone calculated on this basis. The lower values were calculated from data obtained by double isotope derivative analysis of the same stock solution of aldosterone after addition to plasma (see Table IV).

† Per cent recovery is calculated by use of the average of the two values for aldosterone from Column 2.

‡ H^3/C^{14} is the ratio of the cpm of H^3 and C^{14} at the 1,270 voltage setting; see text.

§ Five thousand cpm of corticosterone monoacetate-C^{14} was added initially to provide an artificial contaminant of the sample.

TABLE IV

Reproducibility of aldosterone determinations in plasma

Lots of tritium-labeled acetic anhydride	Aldosterone in each sample	Average value for aldosterone for each lot of acetic anhydride
	μg./ml.	μg./ml.
1	.063, .071, .072, .062	.067
2	.076, .069	.073
3	.079, .081, .077	.079
4	.077, .082, .075	.078
5	.103, .082, .092, .080, .100	.091
6	.080	.080
Mean = 0.079 ± .011 S. D.		Mean = .078 ± .008 S. D.

analyses. The samples were then oxidized with chromic trioxide as for aldosterone except for only five minutes; corticosterone monoacetate was converted to 11-dehydrocorticosterone acetate. The first solvent system was used for the third chromatography and the chromatogram was developed 14 to 16 hours. The ratio of H^3/C^{14} was unchanged after the second chromatography. The data indicate that only two chromatographies were needed for purification at the concentrations of corticosterone observed in the present study.

Data on reproducibility are presented for duplicate samples of plasma aldosterone analyzed concurrently (Table III); the agreement is satisfactory. Similar results were obtained for corticosterone. Additional data on reproducibility have been obtained by analyzing one sample of plasma for aldosterone with each weekly group of 10 to 15 samples of adrenal vein plasma from an experiment. A pool of 150 ml. of peripheral plasma to which aldosterone had been added was the source for each control sample which was analyzed weekly. Six different lots of tritium-labeled acetic anhydride were used and analyses were performed weekly over a period of five months. The results are presented in Table IV. The mean value for the 18-aldosterone determinations with six lots of acetic anhydride was 0.079 ± 0.011 μg. per ml.

RESULTS

Cross-circulation of blood from dogs with thoracic caval constriction into normal recipients via the femoral vessels

Aldosterone secretion increased in two of the three recipient dogs following cross-circulation of blood from a donor dog with thoracic caval constriction (Table V). During study of the first pair of animals, acute constriction of a normal dog which became the donor resulted in a 175 per cent increase (comparison of average control with average experimental value) in aldosterone secretion while a 251 per cent elevation in aldosterone production occurred in the recipient animal. In the second pair of dogs, aldosterone secretion in the chronic donor animal with thoracic caval constriction was markedly elevated above the average rate of aldosterone secretion for normal dogs (Table V, Figure 2); during cross-circulation, aldosterone secretion was not altered appreciably in the donor. In the normal recipient dog of this pair, a 146 per cent increase in aldosterone secretion occurred during cross-circulation. Failure of a response to occur in the third recipient may be a reflection of the abnormally high control values of 0.077 to 0.142 μg. per minute in the recipient; the initial hematocrit in this animal was 39 per cent which was considerably lower than the hematocrit for the other dogs. It seems likely that the initial high values for aldosterone resulted from intra-abdominal bleeding.

The alterations in corticosterone secretion were slight and variable. In the first pair of dogs, the values of corticosterone secretion in the recipient were 33 per cent higher during cross-circulation than during the control period. Also, in the donor a similar slight increase in corticosterone secretion occurred. In the other four animals, corticosterone secretion was either unchanged or fell slightly in both donor and recipient.

The changes in adrenal blood flow after acute

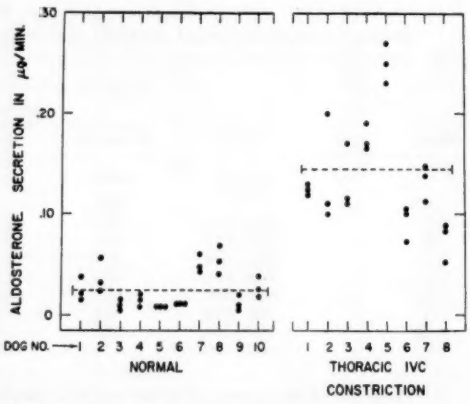

FIG. 2. THE RATES OF ALDOSTERONE SECRETION IN ADRENAL VEIN PLASMA FROM THE RIGHT ADRENAL GLAND OF NORMAL DOGS AND FROM DOGS WITH CHRONIC THORACIC INFERIOR VENA CAVA (IVC) CONSTRICTION

The three determinations for each animal were made at 30 minute intervals.

TABLE V

Cross-circulation via the femoral vessels

Dog pairs	Donor dogs								Recipient dogs							
	Control period			Experimental period					Control period			Experimental period				
	1	2	3	1	2	3	4	5	1	2	3	1	2	3	4	5
							Aldosterone secretion (μg./min.)*									
1†	.004	.021	.034	.054	.071	.054	.052	.043	.005		.004	.013	.019	.011	.019	.017
2	.137	.118	.147	.082	.089	.112	.131		.019	.005	.005	.023	.017	.027	.028	
3	.226	.251	.266	.176	.224	.236	.351		.142	.077	.130	.121	.078	.087	.071	
							Corticosterone secretion (μg./min.)*									
1	0.6	1.1	1.5	1.7	1.8	1.5	1.8	1.6	1.2		1.0	1.3	1.6	1.4	1.6	1.4
2	5.7	5.5	5.1	4.0	4.0	4.0	4.3		2.2	2.3	2.1	2.1	2.4	2.4	2.7	
3	4.3	3.6	3.8	3.9	3.7	3.4	3.4		2.0	2.7	2.6	2.0	1.7	1.7	1.3	
							Adrenal blood flow (ml./min.)‡									
1	3.66	3.10	2.86	2.73	3.06	2.79	3.00	2.17	2.78	3.16	2.25	2.58	2.55	2.50	2.47	1.78
2	4.84	4.08	3.57	3.16	3.33	3.63	3.64		2.98	2.86	2.56	3.68	4.08	3.88	3.64	
3	4.87	4.70	4.35	3.57	3.47	3.64	3.38		1.79	1.99	2.16	2.42	2.13	1.98	1.29	
							Arterial pressure (mm. Hg)									
1	120	110	100	100	100	100	95	60	130	110	115	110	110	110	105	90
2	110	115	130	125	125	130	140		120	120	125	115	105	105	105	
3	65	70	85	75	80	80	80		140	145	145	125	135	135	130	
							Inferior vena caval pressure (mm. water)									
1	−5	30	15	165	200	190	225	290	45	25	25	25	25	25	20	15
2	150	150	145	195	185	180	170		75	60	65	60	65	70	65	
3	130	120	105	175	135	135	135		70	60	75	70	70	75	50	
							Plasma Na (mEq./L.)§									
1	143	137	141	137	139	143	144	144	143	137	141	137	139	143	144	144
2				153	147	140	142		139		145	153	147	140	142	
3	140	142	144	144	144	145	145		142	140	142	144	144	145	145	
							Plasma K (mEq./L.)§									
1	3.5	3.4	3.9	5.4	3.8	3.9		4.1	3.5	3.4	3.9	5.4	3.8	3.9		4.1
2				2.9	3.0	3.3	3.5		3.0	3.4	3.3	2.9	3.0	3.3	3.5	
3	3.4	3.7	3.8	2.8	2.8	3.0	3.9		2.5	2.5	2.5	2.8	2.8	3.0	3.9	

* Secretion in adrenal vein plasma from right adrenal gland.

† The first pair of dogs was normal during cross-circulation for three periods after which the thoracic inferior vena cava was constricted in one of them. Dogs with chronic thoracic caval constriction and ascites were used as donors in Dog Pairs 2 and 3.

‡ From right adrenal only.

§ The concentrations of plasma Na and K were determined from the peripheral blood of one dog only during cross-circulation; in the donor of the second pair of animals, no electrolyte measurements were made before cross-circulation.

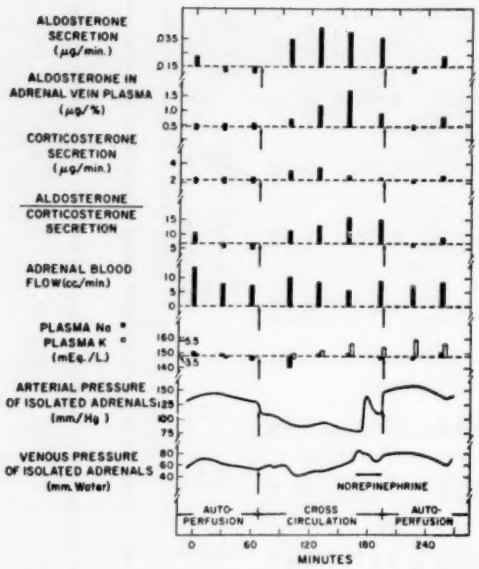

FIG. 3. THE EFFECTS OF CIRCULATION OF BLOOD FROM A DOG WITH CHRONIC THORACIC CAVAL CONSTRICTION INTO THE ISOLATED ADRENALS OF A NORMAL RECIPIENT ANIMAL (DOG NO. 7 IN TABLE I)

The actual $\frac{\text{aldosterone}}{\text{corticosterone}}$ ratio is multiplied by 10^3.

caval constriction or after establishment of cross-circulation were slight and apparently were without effect on hormone secretion (Table V). Inferior vena caval pressure increased in the donor dog only in the first pair of animals, and the high venous pressure present in the other donor dogs with chronic thoracic caval constriction increased further during cross-circulation while venous pressure in the recipient animals was maintained at the control level. Only slight fluctuations in arterial pressure occurred. No consistent alterations in plasma Na or K were detected.

Circulation of blood from dogs with chronic constriction of the thoracic inferior vena cava through the isolated adrenals of normal recipient animals

Observations were first made on a group of eight dogs with chronic thoracic caval constriction, ascites and marked Na retention to determine by the present radioisotope technique the magnitude and consistency of hypersecretion of aldosterone. The data that were obtained are compared (Figure 2) with those reported previously (5) for normal dogs. Aldosterone secretion was elevated in every animal with caval constriction and the mean rate of aldosterone secretion of 0.146 μg. per minute for dogs with caval constriction was sixfold greater than the average rate of 0.024 μg. per minute for normal dogs ($t = 5.8$; $p < 0.001$). It was concluded that the consistency and magnitude of the hyperaldosteronism was sufficient to justify performance of cross-circulation without measurements of aldosterone secretion in the chronic donor dogs.

Seven cross-circulation experiments were conducted using dogs with chronic thoracic caval constriction as donors (Table I). After three control determinations during autoperfusion of the recipient's isolated adrenals, cross-circulation was established with a dog with chronic thoracic caval constriction. In five of the seven experiments, a recovery period with autoperfusion was obtained.

Aldosterone secretion increased in every instance (average increase was 129 per cent) and returned to the control level during the recovery periods (Table I, Figure 3). A statistical analysis of variance showed that the increase was significant at the 1 per cent level ($F = 14.50$, $t = 3.80$). The increase in aldosterone secretion was associated with a greater increase percentage-wise (196 per cent) in the concentration of aldosterone in adrenal vein plasma. Since blood from the donor dogs perfused isolated adrenal glands, the data show a direct effect upon the recipient's adrenals of a humoral agent in donor blood. The rate of corticosterone secretion was slightly higher during cross-circulation in three of the five experiments and fell during the recovery period. Variations in adrenal blood flow occurred and the low values for aldosterone secretion for the third determination during the cross-circulation period in Dogs 4, 5 and 6 were associated with low adrenal blood flows (Table I). Arterial pressure was slightly lower during cross-circulation than during control and recovery periods unless the pressure was maintained by a norepinephrine infusion. Inferior vena caval pressure was unchanged or slightly reduced during cross-circulation and slightly elevated during recovery. No consistent changes in plasma Na were detected during cross-circulation. Plasma K was un-

changed or increased slightly during cross-circulation; in one instance (Figure 3) the slight increase continued during recovery.

Circulation of blood from normal dogs into the isolated adrenals of normal recipient animals

To determine the effects of cross-circulation *per se* on adrenocortical function, eight studies were performed by circulation of blood from a normal animal into the isolated adrenals of a normal recipient. Aldosterone secretion remained unchanged or decreased in all but two dogs and the mean values for aldosterone secretion were unchanged (Table II). During recovery, aldosterone production was slightly elevated in some instances. The variable response is probably attributable to factors producing variation in aldosterone secretion in the normal donors. Corticosterone output was increased slightly (four dogs) or unchanged (four dogs) during cross circulation (Table II, Figure 4) and decreased during recovery. Adrenal blood flow was not appreciably different during the control and cross-circulation periods but was slightly reduced during recovery. The slight decline in arterial pressure was similar to that observed during cross-circulation of blood from dogs with secondary hyperaldosteronism into normal isolated adrenals. Inferior vena caval pressure was unchanged during cross-circulation but was frequently slightly elevated during recovery. In one experiment (Figure 4), the thoracic inferior vena cava was constricted in the normal donor after three determinations had been made during cross-circulation; a 100 per cent increase in aldosterone secretion resulted. The data for arterial and venous pressures and adrenal blood flow on this animal are presented in Table II (see Dog No. 12).

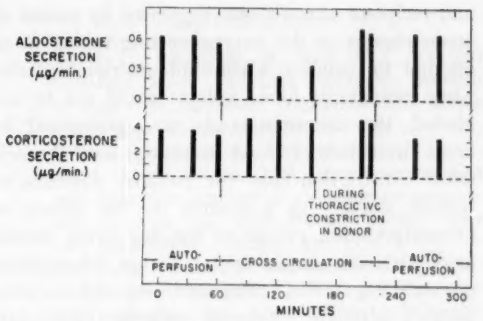

FIG. 4. ALDOSTERONE AND CORTICOSTERONE SECRETION FROM NORMAL ISOLATED ADRENALS BEFORE (AUTOPERFUSION) AND DURING CROSS-CIRCULATION OF BLOOD FROM A NORMAL DOG

Subsequently, the thoracic inferior vena cava in the normal animal was constricted. A recovery period was obtained with the recipient's blood (autoperfusion).

DISCUSSION

The mechanisms regulating aldosterone secretion by the adrenal cortex may be neural, humoral or both. That neural connections to the adrenal gland are unnecessary for aldosterone secretion and maintenance of electrolyte metabolism was suggested more than two decades ago by Levy and Blalock (7). They transplanted one adrenal gland to the neck and removed the other adrenal; several animals lived for months in a normal healthy condition. More recently, Fleming and Farrell (8) showed that acutely transplanted adrenals secrete normal or above normal amounts of aldosterone. Additional data were provided by McDonald, Goding and Wright (9) from chronic studies in unanesthetized sheep. Unilaterally adrenalectomized sheep with the remaining adrenal transplanted to the neck showed maintenance of normal plasma electrolyte concentrations and a normal response to Na depletion as indicated by a marked drop in the salivary Na : K ratio. If a humoral mechanism is involved, it might be an alteration in the concentrations of plasma electrolytes, or it might be hormonal. Slight fluctuations in plasma Na and K occurred in the present study but no consistent changes were detected. Measurements of total plasma osmolality in previous studies of dogs with acute thoracic inferior vena cava constriction and hyperaldosteronism (5) showed no change.

The present experiments demonstrate that blood from dogs with secondary hyperaldosteronism stimulated aldosterone secretion in normal recipient animals and in the isolated adrenals of normal dogs. The increase was associated with an elevation in the concentration of aldosterone in adrenal vein plasma. During the preliminary study of the effects of cross-circulation via the femoral vessels, the volume of blood in both donor

and recipient animals was regulated by means of screw clamps on the interconnecting tubing in an attempt to maintain a constant arterial pressure. Since changes in blood volume could not be excluded, the definitive study was performed by cross-circulation of blood from dogs with thoracic caval constriction into the isolated adrenals of normal dogs. As a control on the effects of cross-circulation *per se* or for any event associated with this experimental design, blood from normal dogs was circulated through normal isolated adrenals. Arterial pressure fell slightly during cross-circulation in most experiments but the decline in pressure was essentially the same for both experimental (Table I) and control (Table II) groups. The arterial pressure in the donor animals was the same as the recorded pressure of blood which perfused the isolated adrenals since the blood pressure in the isolated aorta was derived entirely from blood from the donor. Aldosterone secretion increased in every instance and the change was highly significant during cross-circulation of blood from hyperaldosteronemic donors into isolated adrenals, whereas cross-circulation of blood from normal animals produced no consistent alteration in aldosterone secretion and the mean control and mean experimental values were the same. The present study demonstrates, therefore, the existence of an aldosterone stimulating agent in blood of dogs with experimental secondary hyperaldosteronism and it is suggested that this humoral agent is a hormone. The data agree with the suggestion of Orti, Ralli, Laken and Dumm (10) that a humoral substance provides the stimulus to aldosterone production. Orti and associates reported evidence from studies of rats on a low Na diet that a substance in urine increased aldosterone excretion in feces.

In most of the present experiments, corticosterone secretion was measured to examine the possibility that the circulating hormone is ACTH. Corticosterone rather than hydrocortisone was studied because of the similar polarity of corticosterone monoacetate and aldosterone diacetate during descending chromatography; these two steroids were separated with the same solvent system during the first chromatography. In the dog, the effects of ACTH on corticosterone and hydrocortisone release are very similar. Farrell, Rauschkolb and Royce (11) showed similar marked drops in corticosterone and hydrocortisone after hypophysectomy and an increase in both steroids after ACTH administration. This marked drop in corticosterone after hypophysectomy in the dog and the response to ACTH have been confirmed (Reference 12, and unpublished observations). Also, in an earlier report (5), concurrent measurements of Porter-Silber reacting steroids and corticosterone in adrenal vein plasma showed similar directional changes in every instance. Available evidence indicates, therefore, that alterations in either corticosterone or hydrocortisone secretion may reflect changes in circulating ACTH in the dog.

Increased circulating ACTH alone does not appear to explain the observed increase in aldosterone output. Aldosterone secretion increased markedly in every instance during cross-circulation of blood from dogs with secondary hyperaldosteronism into isolated adrenal whereas corticosterone secretion increased slightly in 60 per cent of the experiments. During the present recovery observations, aldosterone secretion was at the control level or slightly elevated whereas corticosterone output declined further and in many instances this decline was associated with a decrease in adrenal blood flow. In one experiment (Figure 4) in which blood from a normal dog was circulated through normal isolated adrenals, aldosterone secretion fell while corticosterone output increased; subsequent constriction of the thoracic inferior vena cava of the normal donor resulted in a 100 per cent increase in aldosterone production but corticosterone secretion was unchanged. Also, the alterations in corticosterone secretion were similar during cross-circulation of blood from normal dogs into isolated adrenals to the changes found during cross-circulation of blood from dogs with chronic caval constriction. During previous observations (5), acute constriction of the thoracic inferior vena cava produced an increase in aldosterone secretion while corticosterone output fell. Although these findings suggest different regulatory mechanisms for aldosterone and corticosterone secretion, the data do not exclude an influence of ACTH on aldosterone production. Both aldosterone and corticosterone output may have been increased during the control observations as a result of surgery. Hilton and associates (3) have reported near maximal output of hydrocortisone in the isolated adrenal preparation. Additional stud-

ies are needed to identify this aldosterone stimulating substance and to determine its origin.

SUMMARY AND CONCLUSIONS

To test the possibility of a humoral mechanism in the regulation of aldosterone secretion, cross-circulation experiments were performed between dogs with thoracic inferior vena cava constriction and secondary hyperaldosteronism and normal recipient animals. Cross-circulation was established through the femoral vessels, or the isolated adrenals of normal animals were perfused with blood from a hyperaldosteronemic donor. Control and recovery periods were obtained with the recipient's blood or by cross-circulation of blood from a normal dog. Repeated determinations of aldosterone and corticosterone were made in adrenal vein plasma by the double isotope derivative method. Aldosterone secretion in the recipient or in the recipient's isolated adrenals increased markedly in nine of 10 experiments, and returned to the control level in the isolated adrenals of five dogs with recovery observations. Corticosterone secretion increased only slightly in four of eight experiments. No consistent alterations in the concentrations of plasma Na and K were detected. During cross-circulation of blood from eight normal dogs into the isolated adrenals of normal animals, aldosterone secretion was unchanged or decreased in all but two dogs; a slight increase in corticosterone secretion occurred in one-half of the animals. It is concluded that a humoral agent, possibly a hormone, stimulates the adrenals to secrete aldosterone in dogs with secondary hyperaldosteronism.

ACKNOWLEDGMENTS

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TRANSCORTIN: A CORTICOSTEROID-BINDING PROTEIN OF PLASMA. II. LEVELS IN VARIOUS CONDITIONS AND THE EFFECTS OF ESTROGENS *

By AVERY A. SANDBERG and W. ROY SLAUNWHITE, Jr.

(From the Departments of Medicine and Biochemistry Research, Roswell Park Memorial Institute, Buffalo, N. Y.)

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Evidence for the existence in human plasma of a protein with high affinity for the binding of corticosteroids, which we have named transcortin, has been recently published from several laboratories (1–5). Even though the concentration of transcortin is considerably lower than that of albumin, the former binds cortisol approximately 6,000 times as strongly as does the latter (5). Therefore, under physiological conditions most of the plasma cortisol has been shown to be bound to transcortin. Transcortin appears to be saturated at plasma levels of cortisol of approximately 30 to 40 μg. per cent (4–5). Some of the characteristics of the corticosteroid-binding protein have been described by Daughaday (2, 6), Bush (3), Upton and Bondy (4) and ourselves (5).

In a previous paper we reported a method for measuring the binding capacity of transcortin in plasma and showed it to be increased in the plasma of pregnant women (5). Since the concentration of transcortin may play an important role in the metabolism and biological effects of cortisol, it appeared worthwhile to ascertain the transcortin capacity in several clinical states. Data are presented in this paper on the transcortin binding capacity in the plasma of newborn infants and their mothers; in normal children of various ages; in patients with cirrhosis of the liver; in patients with miscellaneous diseases; and following the administration of estrogenic substances.

METHODS AND MATERIALS

Maternal and fetal blood was taken into heparinized syringes immediately following delivery from the antecubital vein and the umbilical vein of the placenta, respectively. The plasma was separated from the erythrocytes shortly following withdrawal of the blood. Some of the plasma was stored at − 10° C. The determination of the binding capacity of transcortin was performed and calculated according to methods previously described (1, 5). Unless otherwise indicated, all binding experiments were performed by dialyzing 10 ml. of diluted plasma (1:5 with physiological saline) against 30 ml. of saline containing approximately 0.3 μg. of C^{14}-cortisol. The binding was determined with and without the addition of 1 μg. of cortisol to the saline in order to measure the decrease in the binding caused by the addition of the carrier cortisol. In a previously published paper (5) we demonstrated that under the conditions of our assay the binding of cortisol by plasma was essentially due to transcortin and not to other plasma proteins. Hence, the term "transcortin binding of C^{14}-cortisol" will refer to the percentage of C^{14}-cortisol bound by 10 ml. of diluted plasma, and the term "transcortin capacity" will be used to indicate the decrease in binding caused by the addition of 1 μg. of cortisol in the dialysis conditions outlined above. It should be pointed out that the "binding capacity" will be inversely related to the decrease in the binding caused by the addition of 1 μg. of cortisol, *i.e.*, the less the decrease in the binding, the higher the binding capacity, and vice versa.

Some of the shortcomings of the method used were

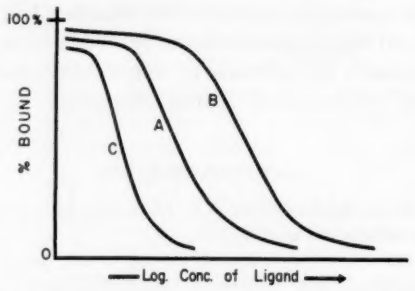

Fig. 1. Representative Theoretical Curves for the Binding of Cortisol (Ligand) by Transcortin

Curve A is assumed to portray conditions under normal concentrations of plasma transcortin; Curve B under increased transcortin levels; and Curve C under decreased transcortin concentration. It is to be noted that with each individual curve the concentration of transcortin is constant, whereas that of cortisol is shown to increase.

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TABLE I

*Transcortin binding of C^{14}-cortisol and capacity in the plasmas of mothers and their newborn infants**

Subject	Mothers		Newborn infants (cord blood)	
	% of C^{14}-cortisol bound	Binding capacity†	% of C^{14}-cortisol bound	Binding capacity†
Sc.	92	14	45	1
Cu.	92	11	63	7
Ja.	87	14	74	22
Ko.	91	13	66	20
Fo.	92	11	72	17
Ma.	87	11	65	19
Ki.	94	11	66	32
Pe.	79	13	57	24
Am.	90	7	58	14
Ra.	91	8	66	
Ru.	90	11	61	14
Sl.	86	7	72	16
We.	86	8	75	21
Hu.	89	10	66	17
Su.	90	11	64	22
Ca.			66	
Ba.	80	9	55	18
Ch.	79	7	66	9
Sa.	87	9	58	
Means±S. D.	88±4.6	10±2.3	64±7.2	17±7.3

* The binding of 0.3 μg. of C^{14}-cortisol and the change, shown as the binding capacity, upon the addition of 1.0 μg. of cortisol; dialysis of plasma diluted 1:5 with saline against saline.

† It should be noted in this and in the following tables, that the binding capacity is inversely related to the values given in the column.

discussed previously, but it would appear that this offers, at present, the best approach to the measurement of transcortin capacity (5). The major shortcoming of the method is the inability to measure the actual concentration of transcortin in the plasma. Nevertheless, knowing three values (the initial binding of C^{14}-cortisol, the binding capacity, and the endogenous cortisol concentration), one can locate reliably the point on one of the binding curves (Figure 1) at which a particular experiment was performed. Since the determination of endogenous cortisol concentrations is impractical, we have relied on 17-hydroxycorticosteroid concentrations (17-OHCS) as approximations of cortisol levels, taking the values either from the literature or from our own data.

The radioactivity in the samples and the plasma 17-OHCS were determined by methods previously described (7, 8). The electrophoretic pattern of the plasma proteins was determined in a Perkin-Elmer Model 38 electrophoresis apparatus. All samples were dialyzed to equilibrium in phosphate buffer, pH 8.6 (0.1 M), and the electrophoretic determinations were made according to standard techniques.

RESULTS

The plasma of 10 normal adult males and females (18 to 45 years of age) bound 84 ± 12.4 per cent (S.D.) of the C^{14}-cortisol and showed a decrease in the binding upon the addition of 1 μg. of cortisol of 26 ± 7.6 per cent.

1. Binding capacity in newborn infants and their mothers and in children

In Table I are shown the values for transcortin binding of C^{14}-cortisol and capacity of mothers and their newborn infants. The binding of C^{14}-cortisol exhibited by the mothers' plasmas was higher (88 ± 4.6 per cent) than that shown by the newborn infants' plasmas (64 ± 7.2 per cent). The differences using the method of paired variates were statistically significant ($p < 0.01$). The binding by the plasmas of newborn infants represents the lowest mean value we have observed. The addition of 1 μg. of cortisol resulted in a greater drop of bound cortisol in the plasma of infants (17 ± 7.3 per cent) than of their mothers (10 ± 2.3 per cent), indicating a lower ($p < 0.01$) binding capacity for cortisol in the former.

The determination of the transcortin levels in the plasma of children of various ages revealed

TABLE II

Transcortin binding of C^{14}-cortisol and capacity in the plasmas of children

Subject	Age	% of C^{14}-cortisol bound	Binding capacity
P. P.*	15–3 mos.	95	28
P. P.*	2–3 mos.	91	22
P. P.*	2–4 mos.	93	23
P. P.*	1–4 mos.	90	26
P. P.*	3–4 mos.	88	29
W. D.	4 mos.	92	30
P. P.*	5 mos.	93	28
P. P.*	9–11 mos.	96	17
K. B.	1 2/12 yrs.	96	21
K. F.	1 5/12 yrs.	98	
C. N.	1 10/12 yrs.	91	
P. P.*	1 6/12–2 yrs.	98	18
H. R.	3 6/12 yrs.	96	21
P. P.*	4 2/12–5 yrs.	96	22
C. R.	5 yrs.	95	
P. P.*	5 yrs.	98	24
P. P.*	3–6 yrs.	92	18
P. M.	6 yrs.	97	24
P. P.*	4–7 6/12 yrs.	96	22
K. Mc.	8 yrs.	96	22
J. B.	8 6/12 yrs.	93	
S. A.	9 yrs.	93	28
S. W.	9 yrs.	94	24
R. D.	9 6/12 yrs.	94	22
J. T.	11 5/12 yrs.	97	
D. B.	11 10/12 yrs.	97	21
Means±S. D.		94±2.7	23±3.7

* Pooled plasma.

TABLE III

Changes in transcortin binding of C^{14}-cortisol and capacity during estrogen administration

Subject, diagnosis	Sex, age	Therapy	Days following beginning of therapy	C^{14}-cortisol bound	Binding capacity	Plasma 17-OHCS
				%		μg. %
E. M.	F	Stilbestrol	0	96	29	14
Carcinoma of breast	49	5 mg.	13	98	10	54
		3× daily	20	98	10	69
			40	99	6	60
N. A.	F	Stilbestrol	0	85	24	23
Carcinoma of breast	52	5 mg.	17	96	12	70
		3× daily	26	98	9	66
R. U.	M	Stilbestrol	0	86	27	16
Carcinoma of prostate	60	5 mg.	0	76	26	20
		3× daily	4	98	17	48
			7	98	12	36
			10	98	12	39
			16	98	10	40
H. S.	M	Stilbestrol	0	90	25	10
Carcinoma of prostate	62	1 mg.	0	87	23	14
		3× daily	2	98	20	24
			9	98	16	31
			15	99	13	38
			21	99	11	34
S. W.	M	Ethinyl	0	91	21	14
Myocardial infarction	72	estradiol	13	96	12	53
		0.5 mg. daily				
F. M.	F	Stilbestrol	0	90	23	13
Carcinoma of breast	70	5 mg.	34	98	11	63
		3× daily				
M. M.	F	Stilbestrol	0	95	24	15
Carcinoma of breast	67	5 mg.	29	98	7	48
		3× daily				
M. S.	F	Stilbestrol	0	96	20	18
Carcinoma of breast	57	5 mg.	29	99	10	44
		3× daily				

significantly ($p < 0.05$) higher binding of C^{14}-cortisol (94 ± 2.7 per cent) than in normal adults. The transcortin capacity (23 ± 3.7 per cent) fell within the normal range for adults. The ages of the children ranged from six weeks to nearly 12 years. In many cases it was necessary to pool the plasmas of several children. No significant difference was observed in the transcortin binding of C^{14}-cortisol and capacity among the various age groups studied (Table II). Unfortunately, we do not have data on transcortin levels in the age group of 12 to 18 years.

2. Effects of estrogen administration on the binding capacity

In previously published work we demonstrated increased transcortin binding of C^{14}-cortisol and capacity during pregnancy, especially the last trimester (5). To ascertain whether these increased transcortin levels might in some measure be due to the increased circulating levels of estrogens, several subjects were given stilbestrol or ethinyl estradiol and the plasma transcortin levels and the 17-OHCS were measured. The data are shown in Table II. It is readily apparent that the administration of the estrogen caused an increase in the transcortin binding of C^{14}-cortisol and, especially, in the transcortin capacity, resulting in the highest values we have observed in our laboratory. It is of interest to note that the transcortin levels increased with time during administration of the drugs. It should be pointed out that the binding capacity is of greater value in evaluating the effect of estrogens on transcortin, since the

transcortin binding of C^{14}-cortisol approached 100 per cent in all the subjects given estrogens and even great variations could not be measured with the presently employed method. On the other hand, the transcortin capacity would reflect more accurately the effects of the estrogen. The plasma 17-OHCS levels rose concomitant with the changes in the binding. The problem as to which occurs first, the rise in the 17-OHCS or transcortin, is now being further investigated.

3. Binding capacity in conditions associated with plasma protein abnormalities

The results in a group of patients with cirrhosis of the liver are shown in Table IV. All the subjects studied were male, except for Patient Ko. (40 year female), and the ages ranged from 32 to 60 years. In general, the transcortin binding of C^{14}-cortisol and capacity were not significantly different ($0.3 < p < 0.4$) from those observed in normal subjects. There appeared to be no correlation between the transcortin levels and the clinical or laboratory findings. In Table V are shown the data obtained in six patients with multiple myeloma. As a group, the transcortin binding of C^{14}-cortisol and capacity of the plasma of these subjects did not differ materially from those seen in normal subjects. There was no apparent correlation between the plasma protein changes and the transcortin levels. In Table VI are shown the transcortin levels in three children with the nephrotic syndrome and in four patients with active rheumatoid arthritis. The children with the nephrotic syndrome had hypoalbuminemia, hypercholesteremia and generalized edema. The transcortin levels are somewhat lower than those observed for normal children of similar age group, but more cases will have to be studied before definite conclusions regarding the transcortin levels in nephrosis can be reached. The transcortin binding of C^{14}-cortisol by the plasmas of the four adult patients with rheumatoid arthritis are somewhat higher than that observed in normal adult subjects, but the binding capacity was within normal limits.

TABLE IV

Transcortin binding of C^{14}-cortisol and capacity in patients with cirrhosis of the liver

Subject	% of C^{14}-cortisol bound	Binding capacity
La.	92	24
Ho.	83	29
We.	83	21
Di.	92	30
Do.	93	28
Fa.	89	24
Pa.	88	22
McL.	90	27
Pi.	86	26
Ma.	88	23
Web.	88	20
Ke.	97	21
Sw.	81	29
Ko.	92	26
Ki.	96	28
Sm.	78	22
Mean±S. D.	89±5.6	25±3.2

TABLE V

Transcortin binding of C^{14}-cortisol and capacity in patients with multiple myeloma

Subject	% of C^{14}-cortisol bound	Binding capacity	Plasma protein (% of total)				
			Alb.	α_1	α_2	β	γ
Go.	79	32	27	7	13	17	35
De.	69	28	14	5	16	13	51
To.	85	28	16	10	8	13	52
Pr.	88	23	23	3	8	15	51
Sl.	83	26	41	14	10	9	26
Sa.	88	30	18	2	3	11	66
Mean±S. D.	82±7.2	28±3.1					

4. Binding of conjugated metabolites of C^{14}-cortisol

Experiments were performed to ascertain the binding of the conjugated metabolites of cortisol

TABLE VI

Transcortin binding of C^{14}-cortisol and capacity in patients with nephrosis and in patients with rheumatoid arthritis

Subject	Diagnosis	Age, sex		% of C^{14}-cortisol bound	Binding capacity
		yrs.			
D. M.	Nephrosis	9	M	81	27
J. L.	Nephrosis	3½	M	89	25
M. R.	Nephrosis	9	M	71	19
W. N.	Rheumatoid arthritis	36	M	93	29
A. B.	Rheumatoid arthritis	65	F	96	24
E. F.	Rheumatoid arthritis	52	F	91	26
M. H.	Rheumatoid arthritis	24	F	92	22

by transcortin, since these conjugates might encroach on the ability of transcortin to bind cortisol. Following the injection of C^{14}-cortisol into a normal male subject, the urine obtained during the initial two hours following the administration of the C^{14}-cortisol was used as a source of conjugated metabolites of C^{14}-cortisol. One ml. of the urine, containing approximately 10,000 cpm, was added either to 5 per cent human serum albumin (HSA) or to saline and dialyzed against *undiluted* plasma. Dialysis was performed for eight days, since preliminary observations indicated that equilibrium was not established between the plasma and the HSA when dialyzed for three days, but did occur in eight days. The binding of urinary C^{14}-cortisol metabolites by the plasma was 1 per cent when the plasma was dialyzed against albumin. It appears, therefore, that there is no special protein which binds the conjugated metabolites of cortisol, comparable to that which binds cortisol. The binding exhibited by the plasma (54 to 59 per cent) dialyzed against the urinary metabolites of cortisol was similar to that found with HSA (9).

Since the amount of cortisol and other steroid metabolites present in the urine sample added to the dialysis system was calculated to be at least 10 μg., and since this amount of steroid metabolites may exceed the binding capacity of the plasma transcortin in the 10 ml. of plasma, the following experiments were performed. Dialysis of diluted (1 : 5) plasma was performed against saline to which 0.3 μg. of C^{14}-cortisol was added. In addition, in another series, 1 ml. of urine from a normal male subject was added to the saline in order to establish the competition of the nonradioactive steroid metabolites of cortisol (and other steroids) with the C^{14}-cortisol for transcortin. In a third experiment some of the urine was extracted with ether, boiled, cooled, then added to the saline, in order to exclude any unconjugated metabolites. Again, the urines used were calculated to contain a minimum of 10 μg. of cortisol metabolites. When no urine was present, 94 per cent of the C^{14}-cortisol was bound by the plasma; when ether-extracted urine was added 84 per cent was bound; and with an original urine 80 per cent was bound. The findings indicate that large amounts of *conjugated* metabolites of cortisol (and of other steroids) in the urine, and, by inference, in plasma, do not compete significantly with the binding of cortisol by transcortin.

DISCUSSION

For a fuller interpretation and understanding of the results presented it may be worthwhile to consider some of the aspects of the binding of a ligand (cortisol) by a protein (transcortin). As shown in Figure 1 the plotting of the percentage of binding against the concentration of cortisol in the presence of a normal amount of transcortin (Curve A) results in the appearance of a sigmoid curve. The two extremities of this curve approach a plateau, indicating that at very low or very high concentrations of cortisol very little change in the percentage of bound cortisol (transcortin capacity) occurs. It is apparent that under the conditions of our experiments, the binding of C^{14}-cortisol occurs somewhere on the descending part of Curve A as evidenced by the greatly decreased binding induced by the addition of 1 μg. of cortisol. Curve B represents the results that prevail when the concentration of transcortin is increased in the plasma. Not only will the percentage of bound cortisol be increased somewhat over that in Curve A, but, more importantly, the transcortin capacity is greatly increased so that 1 μg. of cortisol produces a smaller drop in the percentage of bound cortisol. Curve B approximates the conditions present in pregnancy or following the administration of estrogens. Curve C depicts the conditions when the transcortin concentration is decreased. Under these circumstances, the binding of cortisol, even at normal plasma levels of this steroid, will be greatly reduced and the decrease in the binding induced by extra cortisol will be smaller than that in Curves A or B. Conditions similar to those shown in Curve C are thought to prevail in newborn infants. Since under normal circumstances the plasma concentration of transcortin is small, the lower plateau is approached quickly. As the concentration of cortisol not bound by transcortin increases, its association with the large reservoir of albumin in the plasma becomes of increasing importance.

In previously published work it was shown that the transcortin binding of C^{14}-cortisol and capacity are increased during pregnancy, especially in the last trimester (5), findings compatible with

increased plasma transcortin levels (see Figure 1). Since there is a definite increase in the plasma cortisol during that period of pregnancy, without classical signs of hypercorticism, we advanced the hypothesis that transcortin-bound cortisol was biologically inactive and that the concentration of cortisol in the plasma would have to exceed the effective capacity of transcortin before physiological, and especially pharmacological, effects of cortisol became evident. In the present study the transcortin binding of C^{14}-cortisol in the plasma of the mothers immediately post partum was 88 per cent on the average as compared to 96 per cent obtained during the last trimester. This difference is best explained by the substantial rises in the cortisol levels in women in labor (10, 11) with resultant encroachment on the capacity to bind the added C^{14}-cortisol (see Curve B in Figure 1). Nevertheless, the binding capacity, as shown by the decrease induced by the addition of 1 μg. of cortisol, remained high, especially when compared to that of normal subjects.

From the foregoing discussion it appears that patients with increased levels of plasma transcortin should metabolize C^{14}-cortisol slower than normal subjects. Decreased rates of metabolism of C^{14}-cortisol have been shown to occur in pregnant women (12, 13). It should be emphasized, however, that transcortin may be only one of the factors influencing rates of cortisol metabolism and that the other factors (state of liver function, metabolic rate and so forth) probably play important roles. This is illustrated by the transcortin binding of C^{14}-cortisol and capacity in newborn infants. Even though the plasma 17-OHCS are much lower in the plasma of the newborns (cord blood) immediately post partum than in the plasma of the mothers (10, 11, 14), but on the average similar to those of normal adults, the transcortin binding of C^{14}-cortisol was the lowest observed by us thus far (see Curve C in Figure 1). Since it has been shown that the plasma 17-OHCS decrease to extremely low levels in newborn infants within 24 hours following birth (14), it will be interesting to ascertain the transcortin levels during the first few weeks of life. Upon the addition of 1 μg. of cortisol the binding decreased to very low levels in the newborn infants (mean, 47 per cent, compared to 78 per cent in the mothers, 58 per cent in normal subjects and 83 per cent in the third trimester of pregnancy). Since it has been shown that the concentration of albumin in the plasma of newborn infants is equal to that present in the plasma of children or adults (15), the binding of C^{14}-cortisol exhibited by the plasma of newborn infants, following the addition of 1 μg. of cortisol, can be ascribed to its content of albumin (1). In spite of lower transcortin binding of C^{14}-cortisol, newborn infants have been shown to metabolize cortisol at a slower rate than older children (16, 17). It is possible that the liver is implicated in the slower metabolism of cortisol in the newborn infant.

The transcortin binding of C^{14}-cortisol in children, exclusive of the newborn infants, was somewhat higher than in adult subjects. It is possible that the increased binding shown by the older children's plasma may be due to slightly lower circulating levels of cortisol as inferred from the plasma 17-OHCS concentration (18) and may also explain the normal (23 per cent) drop in the binding upon the addition of 1 μg. of cortisol. Again, for a fuller understanding of the last statement it may be worthwhile to refer to Curve A in Figure 1. It is apparent that the binding of the C^{14}-cortisol by transcortin will be greatly dependent on the concentration of endogenous cortisol in the plasma. At high concentrations of cortisol the binding will be lower down on the curve and the reverse is true for low cortisol levels. Whether the cortisol levels are high or low, but still within the normal range, the binding capacity will be of approximately the same order of magnitude since the initial point of the binding in both cases will be usually located on the upper half of the sloping curve.

Of great interest was the increased transcortin binding of C^{14}-cortisol capacity brought about by the administration of estrogens. We interpret our data to indicate that the drug caused a sharp increase in the concentration of transcortin. This probably explains, in large measure, the ability of subjects given estrogens to tolerate very high levels of cortisol (13, 19). It will be interesting to establish which occurs first, the rise in plasma transcortin or the 17-OHCS concentration. Since it has been shown that the total amounts of corticosteroids produced and excreted in the urine by patients receiving estrogens and in pregnant women are normal or reduced (13, 20, 21), con-

comitant with high plasma 17-OHCS, it can be predicted that the effect of the estrogens or pregnancy is to increase the plasma transcortin levels before the 17-OHCS rise. The increased amounts of transcortin would bind more cortisol and the 17-OHCS in the plasma would rise, due to the unavailability for metabolism of the transcortin-bound steroid. The increased transcortin binding of C^{14}-cortisol capacity in pregnancy may possibly be due to the increased levels of circulating estrogens. The binding capacity increased with time during the administration of the estrogens.

Since the possibility exists that the increased amounts of transcortin induced by estrogen administration originate in the liver, a group of patients with cirrhosis of the liver was examined for transcortin binding of C^{14}-cortisol and capacity. Both were found essentially normal. Two explanations may be advanced for this finding. It is possible that a cirrhotic liver would synthesize lesser amounts of transcortin were it not for the stimulating effect of the increased circulating levels of the estrogens probably present in this condition. The other possible explanation merely states that the liver is not the site of transcortin synthesis. It was with this possibility in mind that several conditions associated with abnormal protein metabolism (multiple myeloma, rheumatoid arthritis, nephrosis) were investigated regarding the transcortin binding of C^{14}-cortisol and capacity in the plasma of these patients. Even though some changes from the normal were seen in the patients, especially those with nephrosis and rheumatoid arthritis and in a few patients with multiple myeloma, in general, the transcortin levels were not greatly divergent from those of normal subjects. Daughaday (*22*) has published the corticosteroid-binding capacity of a relatively small number of patients and has shown it to be decreased in two patients with portal cirrhosis and in one with acute hepatic necrosis. One subject with nephrosis and another with multiple myeloma also had decreased binding capacity. Even though an occasional patient with the above diseases in our studies showed decreased transcortin binding of C^{14}-cortisol and capacity, larger series of these patients will have to be examined before more definite conclusions can be reached regarding the transcortin levels. Special attention must be devoted to nephrosis, since the α_1-globulin levels tend to be low in patients with the syndrome and transcortin is thought to be an α_1-globulin (*2*, 5).

It would appear from the results presented that the various conjugated metabolites of cortisol and other steroids in the urine are not bound by transcortin to any significant extent. Hence, in all probability, the conjugated metabolites in the blood do not interfere with the binding of cortisol.

SUMMARY

1. The plasma transcortin binding of C^{14}-cortisol and capacity, when compared with those of normal adults, are low in newborn infants, possibly increased in children, normal in subjects with cirrhosis of the liver, probably reduced in subjects with the nephrotic syndrome and in some patients with multiple myeloma and slightly increased in subjects with rheumatoid arthritis.

2. The administration of estrogens causes greatly increased transcortin binding of C^{14}-cortisol and capacity in the plasma concomitant with greatly increased levels of the 17-OHCS. Effects of hypercorticism are probably prevented in these subjects by the transcortin binding of cortisol, which is thought to render cortisol biologically inactive and not available for metabolism.

3. The conjugated metabolites of cortisol and other steroids were not bound significantly by transcortin.

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STUDY OF THE PARADOXICAL EFFECTS OF SALICYLATE IN LOW, INTERMEDIATE AND HIGH DOSAGE ON THE RENAL MECHANISMS FOR EXCRETION OF URATE IN MAN *

By T'SAI FAN YÜ and ALEXANDER B. GUTMAN

(From the Departments of Medicine, The Mount Sinai Hospital, and Columbia University College of Physicians and Surgeons, New York, N. Y.)

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Salicylate administered to man in sufficiently large dosage (5 to 6 or more Gm. per day) causes marked uricosuria, characterized by substantially increased urate/inulin clearance ratios attributable to inhibition of tubular reabsorption of the filtered urate. In smaller dosage (1 to 2 Gm. per day) salicylate exerts a contrary effect, retention of urate, associated with lower than normal urate/inulin clearance ratios (1–8). When small doses of salicylate are given concurrently with probenecid, the retention of urate caused by the salicylate is sufficiently pronounced to counteract, to a marked degree, the uricosuric effect of the probenecid (9, 10). Phenylbutazone and phenylbutazone metabolite I similarly cause marked retention of urate when administered in low dosage (7), and are uricosuric in large dosage.

Two explanations of this paradoxical effect have been offered (5, 7, 8). It is conceivable that the tubular transport system effecting reabsorption of urate might be stimulated by small doses and suppressed by larger doses of the drugs in question. Or, a postulated tubular excretory mechanism for urate (not apparent in the usual clearance measurements in man) might be inhibited by small doses, thus causing urate retention; and when larger doses are given tubular reabsorption of urate also is suppressed, the net effect then being uricosuric.

The present study documents the paradoxical action of salicylate by providing more detailed data on the effect of small, intermediate and large doses on the 24 hour urinary urate excretion, using analytical methods incorporating precautions to obviate errors due to the presence of a salicylate metabolite, gentisic acid. The relationships between the renal excretion of salicylate and of urate are then examined by means of simultaneous clearance techniques, including studies under conditions of urine pH made to vary from markedly acid to markedly alkaline. Clearance data on the antagonistic effect of small doses of salicylate on probenecid uricosuria, and vice versa, also are presented. Finally, the implications of the results as a whole are considered in relation to current concepts of renal mechanisms for the excretion of salicylate and of urate, with special reference to inferences regarding the possibility of tubular excretion of urate in man.

METHODS

The subjects selected for investigation were all adult, gouty males, in the intercritical phase of the disorder. Forty-four studies were made in 23 of these subjects to determine the effect of orally administered acetylsalicylic acid, in divided doses totaling 1.0, 2.0, 3.0 and 5.2 Gm. per day, respectively, on the 24 hour urinary urate excretion and the serum urate level. All subjects were maintained on a constant diet low in purines and restricted in protein (60 to 75 Gm. per day) for at least three days preceding study. Twenty-four hour collections of urine were then made for eight or 12 days, blood samples being secured at appropriate intervals. After a four day control period, the patient received the prescribed daily medication for four successive days. The mean daily urinary urate output during this four day medication period, and the serum urate level on the morning of the fourth day of medication, were taken to represent the response to salicylate. Determinations were made also in the four day postmedication period in most instances. Serum and urine salicylate levels were measured in representative cases during the medication period.

Simultaneous urate, salicylate, inulin and para-aminohippurate (PAH) clearance measurements were made in 38 studies in 35 gouty subjects. Standard renal clearance techniques were employed, as previously described in detail (11). In every experiment three premedication inulin and PAH clearance periods, each of 15 to 20 minutes' duration, were first obtained.

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Ten clearance studies were designed to determine the effect of slowly rising and declining plasma and urine salicylate concentrations on the urate clearance. Sodium salicylate was infused slowly at first (16 mg. per minute) for three 10 minute collection periods, then more rapidly for four to seven additional periods, each of 15 to 20 minutes' duration, until a total of 2 to 4 Gm. sodium salicylate had been delivered. Blood samples were procured at the midpoint of each urine collection period. After termination of the salicylate infusion, the collection of (spontaneous) voidings of urine was continued at two hour intervals during the day and four hour intervals during the night, over a period of 20 to 22 hours. Blood samples were taken at the beginning and end of the postinfusion period.

Two of the subjects of the preceding experiments (A. M. and M. M.) excreted a strongly acid urine and the results were included in a study of the effects of low urinary pH on salicylate uricosuria. To amplify these data two additional subjects (S. H. and M. L.) were given ammonium chloride, 16 Gm. in four divided oral doses on the day before the experiment, and 4 Gm. more one hour immediately preceding study. The protocol then followed was the same as in the previous experiments, except for omission of postinfusion urine collections.

Nine clearance studies were designed to determine the effect of alkalinization of the urine, by simultaneous injection of sodium bicarbonate, on salicylate uricosuria. Sodium salicylate was infused slowly in three instances (A. S., J. H., D. R.), as already described. In the six remaining subjects an initial priming dose of 3 Gm. sodium salicylate was given, followed by a sustaining infusion of 0.4 per cent sodium salicylate at a rate of about 4 ml. per minute for 30 to 60 minutes. Urine and blood samples were collected at appropriate intervals. Then, while the sodium salicylate infusion was continued, a priming dose of 7.5 per cent sodium bicarbonate was rapidly injected, the administration of sodium bicarbonate being sustained at a slower rate until a total of 15 Gm. had been delivered. Three or four 15 to 20 minute collection periods were obtained.

Five additional clearance studies were carried out similarly except that acetazolamide, 1.0 Gm., was substituted for sodium bicarbonate to alkalinize the urine.

To determine the effect on urate clearance of alkalinization of the urine *per se*, without prior administration of salicylate, three clearance studies were made with sodium bicarbonate alone, performed in the manner already described, and four with acetazolamide alone, given intravenously (two experiments) or by mouth (two experiments).

The interaction of salicylate and probenecid on urinary urate excretion was first investigated by concomitant oral administration of the drugs, then more precisely by clearance techniques. The latter experiments were initiated by establishment of premedication C_{urate}/C_{inulin} ratios. In four experiments probenecid was first administered by injection of 20 mg. per Kg. body weight within 20 minutes; then a small priming dose (0.3 Gm.) of sodium salicylate was given to establish low plasma salicylate levels, followed by a sustaining solution at accelerating rate until 2.0 to 3.0 Gm. sodium salicylate was delivered over a period of approximately two hours. In four additional experiments the order of drug administration was reversed so as to investigate the effect of probenecid on salicylate uricosuria. A priming dose of 2.0 Gm. sodium salicylate was first rapidly injected; then a sustaining solution of 2.0 Gm. was given more slowly over a period of two hours. At the end of the first hour, an increase in C_{urate}/C_{inulin} having already been established, probenecid, 20 mg. per Kg. body weight, was rapidly injected. Blood and urine samples were procured at appropriate intervals throughout the experiments.

The standard analytical techniques employed for inulin, PAH, urine pH and salicylate in plasma and urine have been indicated elsewhere (11). Plasma and urine urate concentrations were determined in part spectrophotometrically (12), in part by a modification (12) of the colorimetric method of Buchanan, Block and Christman (13), which incorporates uricase digestion and the use of arsenophosphotungstic acid reagent and urea cyanide-carbonate. In this latter method the "true" urate content is determined by difference, the total chromogens minus residual (nonurate) chromogens persisting after incubation of the serum or urine with uricase in borate buffer at pH 9.3. Under ordinary circumstances in both gouty and nongouty individuals, 85 to 90 per cent of the total chromogens are found by this procedure to be "true" urate, and the results check satisfactorily with spectrophotometric methods of uric acid determination (12). However, in subjects receiving large doses of salicylate by mouth the "true" urate values thus obtained by the colorimetric method were found to be too high.

The cause of these discrepancies was investigated, incorporating an additional step into the procedure by incubating samples also in control tubes containing only borate buffer, pH 9.3, without uricase. Ordinarily, no significant difference in total chromogen results, the mean change in 13 urine samples being −0.7 per cent. However, in 12 urine samples from patients given salicylate by mouth the total chromogen content decreased 5.7 to 35.4 per cent (mean fall, 20 per cent). This finding suggested the presence in the urine of appreciable quantities of a chromogenic metabolite of salicylic acid which was unstable in alkaline solution and consequently ordinarily included in the "true" urate fraction. The metabolite in question was isolated by counter-current distribution techniques and identified as gentisic acid, which was found to give as much color with arsenophosphotungstic acid reagent as uric acid (14). Salicylic acid and salicyluric acid are not chromogenic.

In order to estimate the magnitude of the error involved by the presence of gentisic acid, 16 normal and gouty subjects were administered 5.2 Gm. per day of acetylsalicylic acid by mouth. The mean increase in total chromogens in the 24 hour urine, as compared with the premedication excretion, was 369 mg.; of this increase approximately 50 per cent was "true" urate, 42 per cent was in the ether-soluble chromogen fraction (chiefly gentisic acid), and 8 per cent in the aqueous-soluble nonurate chromogen fraction. In individual cases the ether-soluble chromogen constituted 9.5 to 34.4 per cent (mean,

TABLE I

Effect of oral administration of acetylsalicylic acid on urinary urate excretion and serum urate levels in gouty subjects. Potentiating action of sodium bicarbonate

Case	Urinary urate excretion (*mg./24 hrs.*)			Serum urate (*mg. %*)		
	Control	Medication	% Change	Control	Medication	% Change
Medication: Acetylsalicylic acid, 1.0 Gm./day (0.32 Gm. t.i.d.)						
1. O. G.	648	596	− 8	11.4	12.1	+ 6
2. A. R.	766	669	−13	10.8	11.2	+ 4
3. E. M.	620	483	−22	11.2	11.2	0
4. T. T.	358	220	−39	10.9	12.2	+12
Mean			−21			+ 6
Medication: Acetylsalicylic acid, 2.0 Gm./day (0.65 Gm. t.i.d.)						
1. A. R.	543	570	+ 5	9.9	10.4	+ 5
2. C. S.	250	252	+ 1	6.8	7.5	+10
3. J. J.	223	213	− 5	7.8	8.2	+ 5
4. W. L.	836	698	−17	9.0	11.0	+22
5. H. H.	466	378	−19	7.2	7.4	+ 3
6. O. G.	378	278	−26	8.7	8.7	0
7. D. R.	698	417	−40	11.0	11.8	+ 7
Mean			−14			+ 7
Medication: Acetylsalicylic acid, 3.0 Gm./day (1.0 Gm. t.i.d.)						
1. J. J.	192	304	+58	8.7	7.4	−15
2. L. R.	452	675	+49	9.3	7.5	−19
3. J. S.	473	556	+18	8.8	7.3	−17
4. V. P.	224	258	+15			
5. W. L.	686	740	+ 8	8.3	8.5	+ 2
6. C. S.	170	177	+ 4	6.6	6.5	− 2
7. J. D.	999	996	0			
8. M. M.	860	848	− 1	5.0	4.9	− 2
9. L. C.	692	629	− 9	9.4	8.8	− 6
Mean			+16			− 8
Medication: Acetylsalicylic acid, 5.2 Gm./day (1.3 Gm. q.i.d.)						
1. O. G.	343	701	+104	8.2	5.0	−39
2. J. J.	243	462	+ 90	7.7	5.9	−23
3. M. K.	645	1,093	+ 70	10.6	8.7	−18
4. D. R.	545	843	+ 55	10.7	7.4	−31
5. P. M.	547	818	+ 50	5.9	2.2	−63
6. J. D.	761	1,093	+ 44	11.1	9.1	−18
7. R. C.	519	714	+ 38	11.7	7.0	−40
8. V. P.	247	339	+ 37	10.8	10.4	− 4
9. N. W.	633	856	+ 35	10.5	5.3	−50
10. R. B.	357	459	+ 29			
11. R. M.	697	844	+ 21	10.8	5.4	−50
12. J. S.	473	556	+ 18	8.8	8.4	− 5
13. Y. F.	562	540	− 4			
14. P. C.	500	471	− 6	9.2	8.6	− 7
15. C. S.	146	115	− 21	7.0	8.0	+14
Mean			+ 37			−26
Medication: Acetylsalicylic acid + $NaHCO_3$, each 5.2 Gm./day (1.3 Gm. q.i.d.)						
1. R. C.	416	974	+134	12.9	6.4	−50
2. O. G.	318	675	+112	8.8	3.7	−58
3. M. K.	764	1,572	+106	13.2	6.9	−48
4. V. P.	244	450	+ 85	11.5	10.2	−11
5. P. M.	527	892	+ 69	5.9	2.0	−66
6. C. S.	149	243	+ 63	7.0	5.0	−29
7. J. D.	999	1,307	+ 31	12.2	9.7	−20
Mean			+ 86			−40

19.5 per cent) of the total chromogen and was present in quantities representing 11.5 to 78.0 per cent (mean, 31.2 per cent) of the "true" urate. In view of these findings gentisic acid was removed from the urine by multiple extractions with ethyl ether prior to the colorimetric determination of urinary uric acid in all subjects receiving large doses of salicylate (14). The spectrophotometric method of determining uric acid was employed in the later studies since it obviates the need for this preliminary procedure.

The error involved is much smaller in patients receiving 2.0 Gm. per day of acetylsalicylic acid orally: In eight such instances the ether-soluble chromogen constituted a mean of 8.5 per cent of the total chromogen and 11.8 per cent of the "true" urate present. The gentisic acid content of the plasma was found to be too low to warrant correction, even in subjects receiving 5.2 Gm. per day of acetylsalicylic acid by mouth. The same applies to the gentisic acid excreted in the urine in experiments involving rapid intravenous injection of salicylate, as excretion is too rapid to permit of significant metabolic conversion to gentisic acid.

RESULTS

Effect of oral administration of salicylate in low, intermediate and high dosage on 24 hour urinary urate excretion and serum urate levels (Table I, Figure 1)

Acetylsalicylic acid in 1.0 Gm. daily oral dosage caused a decrease in urinary urate excretion per 24 hours of more than 10 per cent of the premedication excretion in three of four subjects studied; the mean *decline* was 21 per cent. This was usually associated with a slight rise in serum urate, the mean increase being 6 per cent. With 2.0 Gm. daily dosage, four of seven subjects showed a decreased 24 hour urinary urate excretion greater than 10 per cent of the control level, none showed a significant increase; the mean *decline* was 14 per cent, associated with a mean increase of 7 per cent in serum urate. In 3.0 Gm. daily dosage there was a distinct reversal of effect. Four of nine subjects excreted more than 10 per cent in excess of their premedication urinary urate output, only one excreted appreciably less urate than before; the mean *increase* was 16 per cent, associated with a mean decline of 8 per cent in serum urate. With 5.2 Gm. daily dosage there was a substantial further increase in urinary urate output per 24 hours, demonstrated in 12 of the 15 subjects tested; the mean *increase* was 37 per cent. The serum urate level was lowered in all but one instance, the mean fall being 26 per cent. Subjects studied at both small and large dosage levels strikingly illustrate the reversible effect of salicylate on urinary urate excretion. Thus D. R., O. G. and J. J. showed *decreases* of 40, 26 and 5 per cent, respectively, in urinary urate output per 24 hours on 2.0 Gm. daily dosage, and *increases* of 55, 104 and 90 per cent, respectively, when given 5.2 Gm. per day.

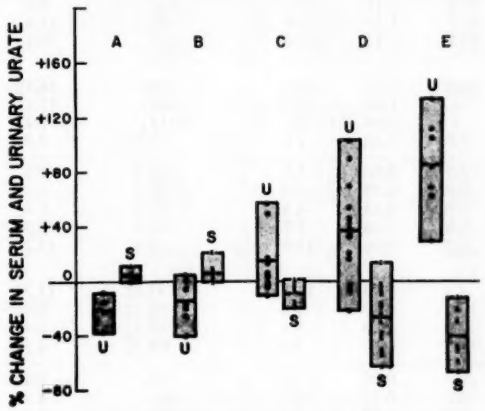

FIG. 1. DISTRIBUTION OF PER CENT CHANGE FROM PREMEDICATION LEVELS OF 24 HOUR URINARY URATE EXCRETION (U) AND SERUM URATE (S) AFTER ORAL ADMINISTRATION OF ACETYLSALICYLIC ACID IN DAILY DOSES OF: A, 1.0 GM.; B, 2.0 GM.; C, 3.0 GM.; D, 5.2 GM; E, 5.2 GM. ACETYLSALICYLIC ACID PLUS 5.2 GM. SODIUM BICARBONATE

The broken lines represent means.

Considerable variation was noted, however, in individual responses to orally administered acetylsalicylic acid at various dosage levels. Some subjects failed to exhibit significant urate retention in 24 hour urine collections on a divided dosage schedule of 1.0 or 2.0 Gm. per day, others failed to show any distinct uricosuric effect even after salicylate doses of 5.2 Gm. per day. A striking example is C. S., a gouty subject with marked renal damage, who showed 21 per cent retention of urate while taking 5.2 Gm. salicylate per day. (These individual variations may account in part for discrepancies in respect to dosage response reported by some prior investigators whose experimental subjects were very limited in number.) The general distribution of responses in 24 hour urinary urate excretion nevertheless indicates that most often in divided oral salicylate dosages between 2.0 and 3.0 Gm. per day there is a distinct reversal from urate retention to uricosuria. At such intermediate

TABLE II

Effect of sustained infusion of sodium salicylate on urate/inulin (respectively, urate/creatinine) clearance ratios

Case	Salicylate			Urate				
	Amount infused (cumulative)	P*	UV†	P	UV	C	C_{in}	C_{ur}/C_{in}
	Gm.	*mg. %*	*mg./min.*	*mg. %*	*mg./min.*	*ml./min.*	*ml./min.*	%
M. L.	0	0	0	9.1	0.88	9.7	129	7.5
	0.23	1.4	0.041	9.3	0.52	5.6	108	5.2
	0.69	2.8	0.24	9.3	0.32	3.4	112	3.1
	1.32	5.7	0.41	9.2	0.37	4.0	98.2	4.1
	1.78	9.0	0.59	8.9	0.62	6.9	102	6.8
	2.89	12.4	0.90	8.9	0.90	10.1	105	9.6
	4.23	17.7	1.73	8.8	1.65	18.8	106	17.7
Salicylate infusion stopped								
0– 4 hrs.		20.2	1.05	8.6	1.23	14.3	85.4‡	16.8§
4– 8 hrs.		18.2	0.98	8.0	1.32	16.5	113‡	14.6§
8–12 hrs.		14.8	0.49	7.8	0.54	6.9	94.0‡	7.4§
12–16 hrs.		12.0	0.41	7.9	0.40	5.1	121‡	4.2§
16–20 hrs.		8.0	0.33	8.4	0.42	5.0	97.2‡	5.1§
J. S.	0	0		11.5	1.69	14.7	128	11.5
	0.34	1.5		11.3	0.90	8.0	125	6.4
	0.53	3.1		10.9	0.77	7.1	125	5.7
	0.75	5.8		10.8	1.04	9.6	121	7.9
	1.05	9.0		10.9	1.46	13.4	118	11.4
	1.40	12.0		10.9	1.96	18.0	125	14.4
	1.81	15.0		10.8	2.34	21.6	116	18.6
	2.21	17.1		10.6	2.89	27.2	115	23.8
	2.71	21.0		10.7	3.37	31.5	119	26.5
Salicylate infusion stopped								
0– 4 hrs.		23.5		10.6	1.74	16.4	96.0‡	17.1§
4– 8 hrs.					1.15	10.9	87.7‡	12.4§
8–16 hrs.					0.61	5.8	91.5‡	6.3§
16–22 hrs.		6.5		10.6	0.33	3.1	118‡	2.6§
S. K.	0	0		11.4	0.71	6.2	120	5.2
	0.63	4.0		11.0	0.39	3.6	128	2.8
	0.92	8.8		11.0	0.49	4.5	112	4.0
	1.27	10.7		11.0	0.70	6.3	117	5.4
	1.62	12.0		10.6	1.11	10.5	114	9.2
	2.19	16.0		10.3	1.32	12.9	96.2	13.4
	3.36	21.8		10.7	2.42	22.6	117	19.4
Salicylate infusion stopped								
0– 4 hrs.		23.8		10.5	1.72	16.4	114‡	14.4§
4–10 hrs.					1.66	16.3	103‡	15.8§
10–16 hrs.					0.87	8.7	113‡	7.7§
16–20 hrs.		9.0		9.9	0.26	2.6	97.6‡	2.9§
M. S.	0	0		8.7	0.50	5.8	89.2	6.5
	0.59	4.5		8.9	0.35	3.9	89.2	4.4
	0.79	7.7		9.0	0.43	4.8	89.0	5.4
	1.22	10.5		8.8	0.68	7.7	97.0	7.9
	1.89	15.5		8.2	1.06	12.9	94.4	13.7
Salicylate infusion stopped								
0– 4 hrs.		19.8		8.3	0.93	11.2	95.8‡	11.7§
4–10 hrs.					0.39	4.6	85.1‡	5.4§
10–18 hrs.					0.48	5.4	85.4‡	6.4§
18–21 hrs.		4.7		9.2	0.35	3.8	88.3‡	4.3§
J. B.	0	0		9.3	0.70	7.5	147	5.1
	0.32	1.8		9.2	0.57	6.2	141	4.4
	0.64	5.3		9.0	0.69	7.7	142	5.4
	0.96	9.5		8.9	0.94	10.5	140	7.5
	1.85	14.9		8.9	1.65	18.6	153	12.2
	3.05	23.0		8.4	2.58	30.8	140	22.0
Salicylate infusion stopped								
0– 4 hrs.		24.2		7.8	1.94	24.8	177‡	14.0§
4– 8 hrs.					1.17	14.8	155‡	9.6§
8–16 hrs.					0.45	5.6	147‡	3.8§
16–21 hrs.		5.4		8.2	0.39	4.7	152‡	3.1§

* P salicylate represents plasma concentration of total salicylate.
† UV salicylate represents urinary excretion of free salicylate.
‡ Values represent C_{cr}.
§ Values represent C_{ur}/C_{cr}.

dosage levels (which, to be sure, may be higher or lower in individual instances) the net effect of salicylate on urinary urate excretion is apt to be negligible.

Upon discontinuance of salicylate medication the enhanced urinary urate excretion per 24 hours of those subjects receiving 5.2 Gm. per day fell sharply, transiently to below premedication levels. In most such cases urate retention in the first 24 hour postmedication period was 15 to 20 per cent of the control excretion (in some instances up to 40 to 50 per cent urate retention), with return to premedication excretion levels within the next 24 hours. In gouty patients with overt renal damage this urate retention phase was apt to be delayed, enhanced urinary urate excretion continuing for the first 24 hour postmedication period, urate retention following within the second 24 hour urine collection period. Patients who received lower oral dosages of salicylate, with no distinct salicylate uricosuria, did not in general exhibit significant urate retention upon discontinuance of medication.

In W. L., a daily regimen of 2.0 Gm. acetylsali-

TABLE III

*Effect of sodium salicylate infusion on C_{urate}/C_{inulin} ratios in the presence of an alkaline urine produced by administration of sodium bicarbonate**

Case	Salicylate			Urate					
	Amount infused (cumulative)	P†	UV‡	P	UV	C	C_{in}	C_{ur}/C_{in}	Urine pH
	Gm.	*mg. %*	*mg./min.*	*mg. %*	*mg./min.*	*ml./min.*	*ml./min.*	*%*	
A. S.	0	0	0	11.0	0.51	4.6	78.0	5.9	6.4
	0.29	2.4	0.19	11.1	0.37	3.3	77.0	4.3	6.4–6.5
	2.12	15.7	1.62	11.2	1.18	10.5	76.9	13.7	6.6–6.7
	4.34§	24.1	7.73	10.6	2.47	23.3	90.0	26.3	7.3–7.6
J. H.	0	0	0	8.5	0.56	6.6	122	5.4	
	0.22	2.5	0.32	8.5	0.48	5.6	120	4.7	
	2.37	12.4	1.07	8.5	0.63	7.4	125	5.9	6.7
	4.33§	17.2	4.40	8.5	1.40	16.5	126	13.1	7.2–7.3
D. R.	0	0	0	11.2	0.95	8.5	140	6.1	
	0.23	2.6	0.26	10.9	0.85	7.8	140	5.6	
	2.03	14.8	1.71	10.4	1.87	18.0	138	13.6	6.9–7.1
	3.65§	18.7	8.47	9.6	4.28	44.6	128	34.9	7.6–7.7
S. L.	0	0	0	10.1	0.50	4.9	114	4.3	5.1
	3.48	27.0	1.04	9.7	1.41	14.5	107	13.6	5.7
	4.38§	26.6	9.19	8.4	2.21	26.3	98.5	26.7	6.9–7.9
L. C.	0	0	0	9.1	0.79	8.6	107	8.0	5.7
	4.00	26.2	1.95	8.9	2.04	22.9	102	22.4	6.3
	5.00§	25.4	10.40	8.4	3.04	36.2	100	36.2	6.9–7.7
B. R.	0	0	0	7.8	0.36	4.7	130	3.6	6.7
	3.54	26.6	3.86	6.0	1.91	31.9	146	21.8	6.7–7.1
	4.30§	25.4	11.52	6.2	2.49	40.2	138	29.1	6.8–7.2
H. W.	0	0	0	9.5	0.58	6.1	85.2	7.2	6.6
	3.58	15.1	3.10	8.7	1.56	17.9	86.2	20.8	6.5
	4.43§	18.9	9.45	7.8	2.43	31.1	85.2	36.5	7.3
H. G.	0	0	0	9.6	0.41	4.3	97.0	4.4	5.5
	4.00	32.4	4.45	8.5	2.21	26.0	94.5	27.5	6.4–6.6
	4.54§	25.2	7.23	8.0	2.12	26.6	93.2	28.6	7.5–7.6
J. S.	0	0	0	8.8	0.41	4.6	85.7	5.4	6.1
	3.67	34.5	3.94	8.5	1.28	15.1	85.3	17.7	6.4–6.5
	4.67§	31.4	11.00	7.7	2.42	31.3	84.5	37.0	7.0–7.3

* Each period indicated represents the mean of at least three urine collection periods.
† P salicylate represents plasma concentration of total salicylate.
‡ UV salicylate represents urinary excretion of free salicylate.
§ Sodium bicarbonate periods.

cylic acid resulted in serum salicylate levels of 3.0 to 5.5 mg. per cent; these low levels were associated with sustained urinary urate retention and a rise in serum urate. In L. C., 3.0 Gm. daily did not yield higher serum salicylate concentrations (3.8 to 5.2 mg. per cent over the four day medication period) and again there was some net urinary urate retention; whereas in J. S., given the same dosage, the serum salicylate rose successively each day from 8.7 mg. per cent (with urate retention on this first day) to 18.5 mg. per cent, the later high levels being associated with distinct uricosuria and fall in serum urate. In patients given 5.2 Gm. acetylsalicylic acid daily the rise in serum salicylate was more rapid and consistent. In O. G., for example, the serum salicylate level reached 12.0 mg. per cent on the first day and was associated on that day with a distinct increase in urinary urate excretion and fall in serum urate; these responses were sustained throughout the four day medication period as the serum salicylate further rose to 17.5 mg. per cent. R. M. responded the same way, although less markedly, as the serum salicylate level rose from 8.2 mg. per cent on the first day to 12.1 mg. per cent on the second day and to 18.0 mg. per cent on the fourth day. In P. C., on the other hand, the rise in serum salicylate was slower, from 9.3 to 10.9 mg. per cent in the first three days during which there was some urate retention, to 14.4 mg. per cent on the fourth day during which there was a substantial increase in urinary urate excretion and fall in serum urate level. The net change in urinary urate excretion over the four day period of medication in P. C. was a decrease of 6 per cent.

Urate/inulin clearance ratios at increasing plasma and urine salicylate levels produced by slow, sustained infusion of salicylate; postinfusion urate/creatinine clearance ratios at declining plasma and urine salicylate levels

The reversible effect of salicylate on urinary urate excretion was defined more precisely by simultaneous clearance measurements in eight slow, sustained salicylate infusion experiments. The results of five experiments are given in Table II; three others are briefly summarized in Table III.

The experiments uniformly revealed an initial

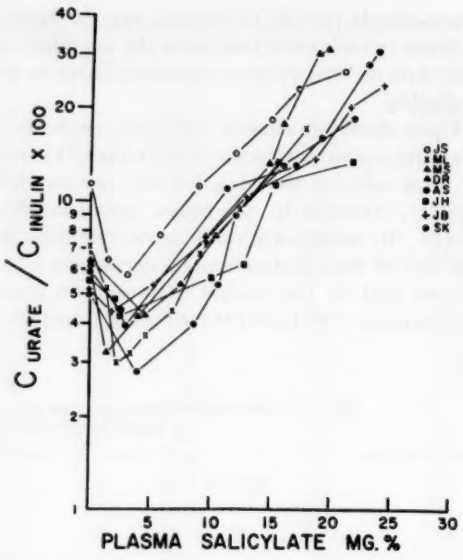

FIG. 2. SEMI-LOG PLOT OF RELATIONSHIP OF C_{URATE}/C_{INULIN} TO PLASMA SALICYLATE LEVELS IN EIGHT SUBJECTS SLOWLY INFUSED WITH SODIUM SALICYLATE

decline in the premedication C_{urate}/C_{inulin} as the plasma and urine salicylate levels slowly rose. This urate retention effect was demonstrable after injection of as little as 230 mg. sodium salicylate, with plasma salicylate levels as low as 1.4 mg. per cent and urinary excretion of free sali-

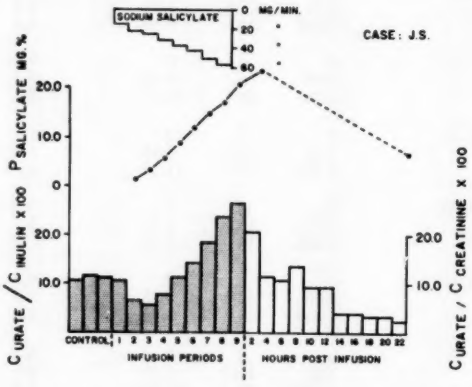

FIG. 3. C_{URATE}/C_{INULIN} (RESPECTIVELY, $C_{URATE}/C_{CREATININE}$) RESPONSE TO SLOWLY RISING PLASMA SALICYLATE LEVELS DURING SLOW INFUSION OF SODIUM SALICYLATE AND DECLINING PLASMA SALICYLATE LEVELS FOR 22 HOURS AFTER THE INFUSION WAS TERMINATED

cylate as low as 0.041 mg. per minute. The decline in C_{urate}/C_{inulin} persisted until the cumulative quantity of sodium salicylate slowly injected totaled a mean of approximately 1.0 Gm. (range, 0.6 to 1.5 Gm.), the plasma salicylate reached levels of 6 to 10 mg. per cent and sometimes more (Figure 2), and urinary excretion of free salicylate was 0.3 to 0.6 mg. per minute. The maximum reduction in C_{urate}/C_{inulin} noted in these experiments averaged 38.5 per cent (range, 13.7 to 58.8 per cent).

As the infusion of salicylate was maintained, C_{urate}/C_{inulin} returned to the control range, and continued to rise to uricosuric levels. Distinctly increased urate/inulin clearance ratios were noted after the cumulative injection of 1.5 to 3.0 Gm. sodium salicylate, with plasma salicylate levels of 10 to 12 mg. per cent or higher (Figure 2), and urinary excretion of free salicylate 0.4 to 0.9 mg. per minute or more. The infusion was terminated without attempting to attain a maximal uricosuric effect.

As the plasma salicylate and urinary excretion of free salicylate levels slowly declined after discontinuance of the salicylate infusion, the same sequence of events was observed in reverse order (Figure 3). For the first two to 10 hours the urate/creatinine clearance ratios remained excessive, then decreased to the premedication range, and continued to decline to values distinctly below the control figures. When observations were discontinued 20 to 22 hours after termination of the salicylate infusion, and the plasma salicylate levels were in the range 4 to 9 mg. per cent, the $C_{urate}/C_{creatinine}$ ratios invariably were still depressed. (The normally low nocturnal excretion of urate accentuates this trend.) These findings correspond with what has been designated "compensatory retention" of urate for a day or two after discontinuance of oral salicylate administration in uricosuric dosage.

These experiments again demonstrate the negligible net effect of salicylate in intermediate dosage on the renal excretion of urate. When salicylate is administered by slow infusion, this intermediate range of total dosage is variable but usually of the order of 1.0 to 1.5 Gm., with plasma salicylate levels usually of the order of 10 mg. per cent (Figure 2), and urinary excretion of free salicylate approximately 0.5 mg. per minute.

Influence of low urine pH on salicylate uricosuria (Table IV)

In subjects with distinctly acid urine, whether spontaneously developed (A. M., M. M.) or induced by administration of ammonium chloride (S. H., M. L.), the retention of urate produced by low dosages of salicylate was particularly pronounced and prolonged, as indicated by a comparison of the data on C_{urate}/C_{inulin} in Tables II and IV. The differences are well illustrated by M. M. (Table IV) whose urinary pH of 6 to 7 during the infusion of sodium salicylate was associated with the expected response in C_{urate}/C_{inulin} (Table II) but who spontaneously developed an unusually acid urine in the postinfusion period. It will be noted that the reduction in C_{urate}/C_{inulin} under these circumstances of distinctly acid urine was unusually marked, in the face of plasma salicylate levels ordinarily associated with a pronounced increase in C_{urate}/C_{inulin}.

The data in Table IV indicate that in subjects with low urine pH a larger than customary cumulative dose of injected salicylate is required to elicit a significant rise in urinary excretion of free salicylate, 3 or 4 Gm. to achieve urinary excretion of free salicylate levels of about 0.6 mg. per minute, whereas at pH levels within the usual range such doses result in UV salicylate levels of 1 to 2 mg. per minute. The corresponding plasma salicylate levels after infusion of 3 Gm. salicylate were 18 to 32 mg. per cent as compared with 12 to 20 mg. per cent. Larger than customary cumulative doses of salicylate, from 2 to more than 4 Gm., were required also to elicit a significant rise in C_{urate}/C_{inulin} above the premedication level. In the case of M. L. even 5.0 Gm. sodium salicylate failed to cause any appreciable increase in urate excretion. No concomitant rise in plasma urate was noted in most instances, presumably because of the short duration of the experiments.

In the systemic acidosis, with acid urine, produced by starvation and by high fat diets there is inhibition of urinary urate excretion (15, 16). In three subjects of this study (not receiving salicylate) given ammonium chloride by mouth to reduce the urine pH to 4.9 to 5.1, a maximal decline of 17 to 37 per cent was noted

TABLE IV

Effect of sodium salicylate infusion on C_{urate}/C_{inulin} ratios in the presence of an acid urine

Case	Salicylate: Amount infused (cumulative)	Salicylate: P*	Salicylate: UV†	Urate: P	Urate: UV	Urate: C	C_{in}	C_{ur}/C_{in}	Urine pH
	Gm.	*mg. %*	*mg./min.*	*mg. %*	*mg./min.*	*ml./min.*	*ml./min.*	%	
S. H.	0	0	0	13.0	0.90	6.9	96.0	7.2	4.5
	0.23	0.6	0.04	13.0	0.81	6.2	84.0	7.4	4.5
	0.35		0.12	13.0	0.47	3.6	105.0	3.4	4.5
	0.50	0.7	0.32	11.8	0.36	3.1	95.0	3.3	4.5
	1.04		0.38	11.8	0.66	5.6	81.0	6.9	4.5
	2.24	19.7	0.45	11.8	0.93	7.9	80.0	9.9	4.5
	3.43	18.1	0.63	11.8	1.30	11.0	92.5	11.9	4.6
	5.00	22.4	1.28	11.8	2.02	17.1	84.0	20.4	4.7
M. L.	0	0	0	9.1	0.84	9.2	135	6.8	4.5
	0.34		0.06		0.33	3.6	133	2.7	4.5
	0.50		0.22		0.27	3.0	125	2.4	4.5
	1.22		0.32		0.36	4.0	139	2.9	4.5
	1.72		0.48	9.1	0.42	4.6	136	3.4	4.5
	2.29		0.43		0.49	5.4	140	3.9	4.7
	2.94		0.43		0.52	5.7	114	5.0	4.7
	3.43	32.5	0.48	9.1	0.65	7.1	124	5.7	4.7
	4.00		0.43		0.66	7.3	130	5.6	4.7
	4.62		0.61		0.85	9.3	124	7.5	4.7
	5.00	31.7	0.61	9.1	0.83	9.1	123	7.4	4.7
A. M.	0	0	0	10.7	0.74	6.9	138	5.0	5.9
	0.15	2.9	0.12	10.7	0.39	3.7	150	2.5	5.6
	0.18	3.6	0.16	10.7	0.41	3.8	160	2.4	5.6
	1.26	9.5	0.24	10.1	0.31	3.1	157	2.0	5.2
	1.70	9.9	0.33	10.2	0.18	1.8	167	1.1	5.3
	2.10	10.3	0.43	10.2	0.56	5.5	179	3.1	5.4
	3.18	20.1	1.69	10.1	2.33	23.1	190	12.2	6.4
	4.10	20.3	2.53	9.7	2.76	28.5	188	15.2	6.7
	Salicylate infusion stopped								
	0– 5 hrs.		2.02	9.6	1.33	13.9	157‡	8.9§	
	5– 9 hrs.		0.81	9.4	1.80	19.2	219‡	8.8§	
	9–17 hrs.		0.35	9.2	0.48	5.2	180‡	2.9§	
	17–19 hrs.	4.0	0.27	9.0	0.34	3.8	152‡	2.5§	
M. M.	0	0	0	8.9	0.55	6.2	114	5.4	6.0
	0.32	1.8		8.8	0.28	3.2	104	3.1	6.5
	0.68	3.4	0.11	8.7	0.33	3.8	109	3.5	5.9
	1.14	6.5	0.26	8.7	0.53	6.1	108	5.6	6.2
	1.56	14.2	0.33	8.7	0.51	5.9	108	5.5	6.4
	2.28	17.5	0.48	8.7	0.66	7.6	108	7.0	6.0
	2.73	20.7	0.72	8.5	1.02	12.0	112	10.7	6.3
	3.57	25.4	1.15	8.3	1.28	15.4	112	13.8	7.0
	4.85	32.0	1.95	7.8	1.78	22.8	113	20.2	7.1
	Salicylate infusion stopped								
	0– 4 hrs.	26.1	0.19	7.7	0.54	7.0	111‡	6.3§	5.1
	4– 8 hrs.	18.5	0.20	7.4	0.50	6.8	112‡	6.1§	5.3
	8–14 hrs.	16.7	0.14	7.1	0.28	4.0	105‡	3.8§	5.2
	14–20 hrs.	15.1	0.23	7.3	0.16	2.2	89‡	2.5§	5.2

* P salicylate represents plasma concentration of total salicylate.
† UV salicylate represents urinary excretion of free salicylate.
‡ Values represent C_{cr}.
§ Values represent C_{ur}/C_{cr}.

in the 24 hour urinary urate output. This is substantially less than the decrease effected by acidification of the urine in subjects receiving salicylate.

Influence of alkalinization of the urine on salicylate uricosuria

a. Sodium bicarbonate. As shown in Table I, the uricosuric effect of acetylsalicylic acid ad-

ministered in 5.2 Gm. daily oral dosage is markedly increased by the simultaneous ingestion of 5 Gm. per day sodium bicarbonate. Except for J. D., the response was uniform, even in otherwise refractory C. S. The mean urinary urate output per 24 hours was increased 86 per cent after alkalinization, as compared with 37 per cent after salicylate alone, and the mean plasma urate level was 40 per cent lower, as compared with 26 per cent.

In two experiments (not included in Table I) a daily oral dosage of 2.0 Gm. acetylsalicylic acid was supplemented with 4 Gm. per day sodium bicarbonate. In J. J., who showed no significant change in urinary urate excretion per 24 hours with salicylate alone, there was no appreciable effect of supplementation with sodium bicarbonate. In D. R., retention of urinary urate decreased from 40.3 to 2.0 per cent, with a corresponding decline in serum urate.

In Table III are summarized the results of nine clearance studies in which a substantial increase in C_{urate}/C_{inulin} was first produced by infusion of sodium salicylate and, while maintaining the plasma salicylate at similar high levels by continued infusion, sodium bicarbonate was then simultaneously injected at rates sufficient to keep the urine pH at 6.8 to 7.9. In every case alkalinization of the urine with bicarbonate resulted in a marked increase in urinary excretion of free salicylate and, except in H. G., potentiation of salicylate uricosuria. The mean C_{urate}/C_{inulin} ratio in the control period was 5.6 per cent; following infusion of salicylate alone, 17.4 per cent; and following infusion of salicylate and bicarbonate, 29.8 per cent. Concomitant injection of sodium bicarbonate thus almost doubled the C_{urate}/C_{inulin} produced by salicylate alone.

b. Acetazolamide. Five similar clearance studies in which the urine was alkalinized with acetazolamide are summarized in Table V. The experimental conditions achieved in respect to increase in urine pH and augmentation of urinary excretion of free salicylate were comparable to those in the preceding studies with sodium bicarbonate (Table III). Except in B. C., however, no increase in C_{urate}/C_{inulin} beyond that produced by salicylate alone, prior to administration of acetazolamide, was obtained; on the contrary, the urate/inulin clearance ratios de-

TABLE V

*Effect of sodium salicylate infusion on C_{urate}/C_{inulin} ratios in the presence of an alkaline urine produced by administration of acetazolamide**

Case	Salicylate			Urate			C_{in}	C_{ur}/C_{in}	Urine pH
	Amount infused (cumulative)	P†	UV‡	P	UV	C			
	Gm.	*mg. %*	*mg./min.*	*mg. %*	*mg./min.*	*ml./min.*	*ml./min.*	%	
H. G.	0	0	0	9.5	0.92	10.1	131	7.7	6.0
	3.67	21.9	5.61	8.7	3.25	37.1	125	29.8	7.1–7.8
	4.96§	22.5	8.13	8.2	1.93	23.6	109	21.7	7.7–7.8
A. J. R.	0	0	0	8.6	0.55	6.3	136	4.7	5.6
	3.78	23.9	1.70	8.6	1.94	22.7	122	18.6	5.7–6.1
	5.24§	23.4	5.65	8.6	0.98	11.4	108	10.7	7.1–7.6
E. F.	0	0	0	5.4	0.68	12.6	143	8.8	6.4
	4.00	24.1	4.65	4.7	2.13	45.0	142	31.7	6.4–6.8
	4.92§	25.9	10.6	4.7	1.44	30.6	110	27.8	7.4–7.7
M. N.	0	0	0	11.5	0.69	8.9	92.9	9.5	6.6
	3.91	21.7	4.38	10.6	2.58	24.3	84.3	29.0	7.1–7.3
	4.93§	23.6	6.05	10.6	1.97	18.6	82.2	22.6	7.5–7.8
B. C.	0	0	0	9.7	0.57	5.8	152	3.9	5.1
	3.80	16.5	0.49	9.7	1.20	12.4	146	8.5	5.3–6.1
	4.92§	16.1	3.53	9.7	1.28	13.2	126	10.5	7.6–7.8

* Each period indicated represents the mean of at least three urine collection periods.
† P salicylate represents plasma concentration of total salicylate.
‡ UV salicylate represents urinary excretion of free salicylate.
§ Acetazolamide periods.

TABLE VI

*Effect of sodium bicarbonate or acetazolamide on C_{urate}/C_{inulin} ratios**

Case	Period	Urate P	Urate UV	Urate C	C_{in}	C_{ur}/C_{in}	Urine pH
		mg. %	*mg./min.*	*ml./min.*	*ml./min.*	*%*	
H. K.	Control	9.1	0.63	6.9	105	6.6	5.8–6.0
	$NaHCO_3$	9.2	0.90	9.8	112	8.7	7.3–7.4
M. L.	Control	10.0	0.71	7.1	104	6.9	5.6–7.7
	$NaHCO_3$	9.8	0.82	8.4	118	7.1	7.3–7.7
H. N.	Control	9.9	1.01	10.2	95.5	10.7	6.5
	$NaHCO_3$	9.6	1.37	14.2	106	13.4	7.2–7.4
A. L.	Control	9.8	1.03	10.5	106	9.9	6.7
	Acetazolamide (0.5 Gm. I.V.)	9.8	0.75	7.3	81.8	8.7	7.5–7.8
R. M.	Control	10.9	1.01	9.3	159	5.8	6.4
	Acetazolamide (1.0 Gm. I.V.)	10.9	0.72	6.6	123	5.4	7.3–7.6
M. W.	Control	9.5	0.52	5.4	128	4.2	4.9
	Acetazolamide (2.0 Gm. P.O.)	9.5	0.37	3.9	100	3.9	6.9–7.5
R. C.	Control	8.7	0.58	6.7	70.6	9.5	5.3
	Acetazolamide						
	(1.0 Gm. P.O.)	8.7	0.31	3.6	50.9	7.1	6.8–7.1
	(2.0 Gm. P.O.)	8.7	0.35	4.0	55.9	6.8	6.6–6.9
		8.7	0.25	2.8	71.0	4.0	7.1–7.8

* Each period indicated represents the mean of three to seven urine collection periods.

clined somewhat. The mean C_{urate}/C_{inulin} in the control period was 6.9 per cent; following infusion of salicylate alone, 22.5 per cent; and following joint administration of salicylate and acetazolamide, 18.7 per cent.

In order to dissociate the effects of alkalinization of the urine *per se* on the urinary excretion of urate, four clearance studies were made after administration of acetazolamide alone, without prior infusion of salicylate (Table VI). The results indicate a mean 32.5 per cent reduction in C_{urate}. This was associated with a decline in glomerular filtration rate (23.3 per cent), in part accounting for the fall in C_{urate}, to give a mean 14.6 per cent lowering in C_{urate}/C_{inulin}. Comparable studies in which sodium bicarbonate was rapidly injected to alkalinize the urine in three subjects (Table VI) revealed a mean rise of 20.8 per cent in C_{urate}/C_{inulin}, from a mean clearance ratio of 8.1 to 9.7 per cent. Sodium bicarbonate given alone in 5 Gm. oral daily dosage had no discernible effect on the 24 hour urinary excretion of urate.

Effect of salicylate in low and high dosage on probenecid uricosuria; effect of probenecid on salicylate uricosuria

The uricosuria induced by probenecid is markedly suppressed by small doses of salicylate (9). Thus L. G. excreted a mean of 486 mg. urate per 24 hours before medication, 824 mg. while taking 2.0 Gm. probenecid daily, 413 mg. during three days while 2.6 Gm. acetylsalicylic acid was added to the probenecid, and 850 mg. when salicylate was discontinued. The corresponding serum urate levels were 7.0, 3.0, 6.3 and 3.9 mg. per cent. In another representative patient, N. W., subjected to the first three phases of the same protocol, the respective figures for mean urinary urate per 24 hours were 399, 1,258 and 690 mg., and the serum urate levels were 9.5, 5.2 and 7.4 mg. per cent.

When large doses of acetylsalicylic acid, 5.2 Gm. per day, were added to the probenecid regimen the uricosuric response was but little affected, except for the first day when the serum

salicylate level was not yet fully established. Thus the basal urinary urate excretion in L. G. (previously cited) rose to a mean of 907 mg. per 24 hours with 2.0 Gm. probenecid daily, fell to 337 mg. on the first day after adding 5.2 Gm. per day salicylate to the regimen, averaged 846 mg. subsequently on combined medication, and 922 mg. when salicylate was withdrawn. The corresponding serum urate levels were 7.6, 4.6, 5.0 and 4.4 mg. per cent. In another representative patient, J. O., the respective figures for mean urinary urate per 24 hours were 483, 640, 480, 668 and 725 mg., and the serum urate levels were 11.5, 7.1, 6.8 and 6.8 mg. per cent.

The clearance studies summarized in Table VII delineate these effects more precisely. In O. G. and S. L., the initial C_{urate}/C_{inulin} ratios increased five- to sevenfold after rapid infusion of probenecid, fell sharply to approximately twofold values as sodium salicylate was slowly infused, then rose again as the plasma salicylate reached levels commonly associated with salicylate uricosuria. The reverse sequence, injection of probenecid after establishment of salicylate uricosuria, caused a modest but fairly consistent decrease in the augmented excretion of urate and a slight decline in the elevated C_{urate}/C_{inulin} ratios produced by salicylate. These changes were associated with a fall in urinary excretion of salicylate and rise in plasma salicylate. There was no indication of any additive effect of the two uricosuric agents.

TABLE VII

*Interaction of sodium salicylate and probenecid on urate/inulin clearance ratios**

Case	Salicylate			Urate				
	Amount infused (cumulative)	P†	UV‡	P	UV	C	C_{in}	C_{ur}/C_{in}
	Gm.	*mg. %*	*mg./min.*	*mg. %*	*mg./min.*	*ml./min.*	*ml./min.*	*%*
O. G.	0	0		8.0	0.42	5.2	106	4.9
	0§	0		7.0	2.06	29.5	82.0	36.0
	0.23	0.6		6.6	1.62	24.5	71.4	34.4
	0.50			6.5	0.97	14.8	92.0	16.1
	0.84	7.8		6.4	0.65	10.2	76.7	13.3
	1.45			6.4	0.64	9.9	67.3	14.7
	1.87	15.0		6.4	1.04	16.3	86.4	18.8
	2.57	20.9		6.4	1.34	20.9	85.0	24.6
S. L.	0	0		9.4	0.89	9.5	145	6.6
	0§	0		9.1	2.22	24.4	137	34.0
	0.20	1.3		8.8	3.24	36.7	112	32.9
	0.49	3.7		8.4	3.12	37.2	142	26.2
	0.80	6.1		8.4	2.59	30.8	167	18.4
	1.17	8.6		8.8	1.49	17.0	150	11.4
	1.55	13.4		8.8	1.32	15.0	150	10.0
	2.23	15.6		8.9	1.79	20.0	148	13.5
	3.00	20.6		8.6	1.57	18.3	107	17.1
I. S.	0	0	0	6.7	0.50	7.5	116	6.5
	2.88	20.5	2.50	6.8	1.82	26.9	123	21.9
	4.12§	23.9	1.98	6.4	1.17	23.3	117	19.9
R. S.	0	0	0	6.7	0.84	6.5	111	5.8
	3.32	21.0	1.37	6.8	0.93	13.7	107	12.8
	4.06§	25.0	1.15	6.7	0.90	13.5	106	12.7
N. B.	3.09	19.9	2.07	7.5	1.40	18.8	82.1	22.7
	3.79§	22.5	1.40	7.0	1.20	17.2	86.9	19.8
L. W.	3.98	24.1	4.30	7.0	2.00	28.6	96.5	29.7
	4.59§	24.7	2.62	6.2	1.68	27.1	97.8	27.7

* Each period indicated represents the mean of at least three urine collection periods for Cases I. S., R. S., N. B. and L. W.

† P salicylate represents plasma concentration of total salicylate.

‡ UV salicylate represents urinary excretion of free salicylate.

§ Probenecid, 20 mg. per Kg. body weight, intravenously as rapid injection.

DISCUSSION

The data define three distinct facets of the action of salicylate on the renal mechanisms for excretion of urate in man. The first discernible effect of salicylate is demonstrated to be suppressive, associated with relatively low concentrations of salicylate in the plasma and urine produced by small doses, such as are commonly employed for general analgesic purposes.[1] Large doses (high salicylate concentrations in the plasma and urine) have a markedly augmentative effect. Betwixt these, in an intermediate zone of dosage, the net effect of salicylate is apt to be negligible; the drug seemingly is virtually inactive in respect to the urinary excretion of urate. The absolute dosage of salicylate required to initiate this sequence varies somewhat from individual to individual, the urine pH being one important variable in this regard, but the relative dosage sequence appears generally to hold in normal and gouty man.

Before understaking an analysis of this paradoxical effect, it would be useful to summarize present knowledge of the mechanisms of renal excretion of salicylate and of urate in man.

Mechanisms of renal excretion of salicylate in man

Approximately one-third of the plasma salicylate is not bound to plasma proteins in man (11) and is presumed to be filtered at the glomerulus. The subsequent fate of the filtered free salicylate is modified by the urine pH. Under the ordinary circumstances of slightly acid urine, $C_{\text{free salicylate}}/C_{\text{inulin}}$ is of the order 0.2 to 0.4 (11), implying tubular reabsorption of the filtered free salicylate. Of the salicylate appearing in acid urine, only a small part (about 20 per cent or less, depending on the urine pH) is excreted as free salicylate. The major proportion is in conjugated form (18), combined with glycine as salicyluric acid (55 to 65 per cent) and with glucuronic acid as salicyl acyl and phenolic glucuronides (20 to 30 per cent) (18, 19). Approximately 20 per cent of ingested salicylate cannot be recovered in the urine as salicyl compounds because of metabolic conversion to gentisic acid and other degradation products (18).

If the urine is made distinctly alkaline, whether by administration of sodium bicarbonate (11, 19–25) or acetazolamide (25), or as a result of overbreathing (25), the rate of urinary excretion of salicylate is greatly enhanced, and the proportion of free salicylate increases to 60 to 90 per cent or more of the total urinary salicylate. This marked rise in $C_{\text{free salicylate}}$ is not due to any increase in the filtered load of free salicylate, and hence is ascribable to diminution of tubular reabsorption. $C_{\text{free salicylate}}/C_{\text{inulin}}$ may exceed 1.0 if the urine is made sufficiently alkaline, indicating tubular excretion of free salicylate in man (11, 19, 23, 25).

In view of the striking urinary pH dependence of the renal excretion of salicylic acid (pKa about 3.0), "passive" tubular transfer processes, dependent upon the pH gradient between the tubular urine and the peritubular fluid, may be assumed to play an important role in both tubular reabsorption and excretion of free salicylate (25–27). However, tubular transfer of free salicylate is not altogether attributable to nonionic diffusion, since probenecid causes a substantial reduction in urinary excretion of free salicylate with a corresponding rise in plasma salicylate, irrespective of the urine pH (11, 19). This effect of probenecid is reminiscent of its action on the renal excretion of penicillin and other compounds "actively" secreted by the tubules. "Active" tubular secretion of free salicylate is further implied by transfer against an H^+ gradient, as indicated by its (limited) reabsorption in alkaline urine and suspected excretion in acid urine (11, 19, 23). Tubular secretion of salicyluric acid (28) and of salicyl acyl and phenolic glucuronides (19) has been clearly demonstrated by Schachter and Manis (19), and occurs at a rate independent of the urine pH, but determined chiefly by the rate of conjugation, largely in the liver (19).

Mechanisms of renal excretion of urate in man

The filtered urate load, calculated on the basis of complete filtrability of plasma urate at the glomerulus (29, 30), greatly exceeds the quantity of urate appearing in the urine. The deficit,

[1] As mentioned elsewhere (12), the hyperuricemia thus resulting from ingestion of salicylate in low dosage is not uncommonly encountered and is frequently misconstrued; when associated with joint pains it is sometimes attributed to gout. Even formal studies of the interpretation of hyperuricemia may neglect this iatrogenic etiology (17).

usually 90 to 95 per cent of the filtered load in normal and gouty man (31, 32), indicates tubular reabsorption. The tubular capacity for reabsorption of urate has been shown to be limited, of the order of 15 mg. per minute in normal man (31), but nevertheless much larger than the urate load delivered to the tubules in normal and most gouty subjects under ordinary circumstances (31, 32).

The conventional interpretation of urate clearance data in man limits the role of the kidney to glomerular filtration and tubular reabsorption of urate. The urate appearing in the urine is considered to represent that 5 to 10 per cent of the filtered urate load which has escaped reabsorption (Figure 4). Another possible interpretation, also consistent with the clearance data, considers tubular reabsorption of the filtered urate to be virtually complete under ordinary circumstances, and the urinary urate to be derived by tubular excretion (7, 32, 33) (Figure 4), *i.e.*, a mechanism similar to that postulated for potassium (34, 35) and thiosulfate (36). The processes of tubular reabsorption and excretion of urate both are conceived to be chiefly "active," in view of their limited capacity and the inhibiting effects of a variety of chemical agents. Nonionic diffusion of uric acid (pKa 5.4) appears to play a minor role in tubular transfer. While the excretion of urate is diminished in acid urine and slightly increased in urine made alkaline by the administration of sodium bicarbonate, alkalinization of the urine by use of acetazolamide leads to some decrease in renal excretion of urate (37). The contrary effects of alkalinization of the urine with bicarbonate and acetazolamide indicate that, in contrast to the nonionic diffusion of other weak acids (27), the tubular transfer of urate is not greatly influenced by alkaline urine pH alone.

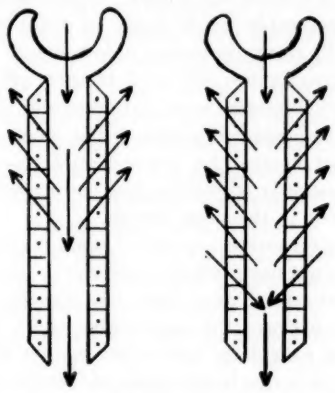

FIG. 4. SCHEMATIC REPRESENTATION OF TWO POSTULATIONS OF RENAL REGULATION OF URIC ACID EXCRETION IN MAN

On the left, the plasma urate is pictured as virtually wholly filtrable at the glomerulus, the filtered urate is largely but not completely reabsorbed in passage through the tubules, and the excreted urate represents that small fraction of the filtered urate which has escaped reabsorption. On the right, the plasma urate again is pictured as virtually wholly filtrable at the glomerulus, the filtered urate is completely reabsorbed in the tubules, and the urate appearing in the urine is secreted by the tubules. This figure originally appeared in Bull. N. Y. Acad. Med. 1958, **34**, 287 (33).

Relationship of concentration of free salicylate in the tubular urine to the tubular regulation of urate excretion

To return now, in the light of the foregoing résumé, to the primary consideration of this study, a critical point in interpretation of the paradoxical effects of low, intermediate and high dosages of salicylate is whether they are related to the corresponding salicylate concentrations in the plasma or in the tubular urine. This would be difficult to establish under ordinary circumstances, in which the rate of renal excretion of salicylate parallels the plasma salicylate concentration. However, dissociation of this relationship can be effected by rendering the urine distinctly acid or alkaline. In distinctly acid urine, urinary excretion of free salicylate remains lower than ordinarily, even after relatively large doses of salicylate, and the plasma salicylate rises inordinately. Under these circumstances the rise in C_{urate}/C_{inulin} is found to be delayed and less pronounced. This effect on C_{urate}/C_{inulin} ratios is distinctly greater than obtained by acidification of the urine *per se* without salicylate. When the urine is made distinctly alkaline with sodium bicarbonate, urinary excretion of free salicylate is greatly augmented and the plasma salicylate rapidly falls unless constantly replenished (as in the infusion experiments cited). Under these circumstances C_{urate}/C_{inulin} is found to increase, significantly above the ratios obtained solely by alkalinization of the urine

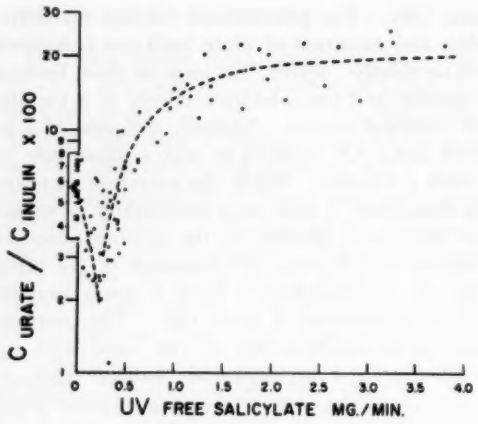

FIG. 5. SEMI-LOG PLOT OF RELATIONSHIP OF C_{URATE}/C_{INULIN} TO URINARY EXCRETION OF FREE SALICYLATE IN 14 SUBJECTS SLOWLY INFUSED WITH SODIUM SALICYLATE

The curves are schematically drawn by inspection. The points include measurements in subjects with markedly acid urine. Points representing measurements in markedly alkaline urine extend the abscissa to 14 mg. urinary excretion of free salicylate per minute and have therefore been omitted; the points lie slightly above the extended curve for urine alkalinized with sodium bicarbonate, slightly below for urine alkalinized with acetazolamide (see text).

with bicarbonate without prior administration of salicylate. When acetazolamide is used to alkalinize the urine in patients receiving salicylate, the excretion of free salicylate again is accelerated, and the plasma salicylate falls unless constantly replenished. However, C_{urate}/C_{inulin} is not increased in this instance, but declines somewhat below the levels reached by acetazolamide without prior administration of salicylate.

The combined results of experiments using ammonium chloride, sodium bicarbonate or acetazolamide indicate that salicylate exerts its various effects on urate excretion largely at the renal tubular level, although these effects seem to be modified by the state of metabolic acidosis or alkalosis. Further, the paradoxical effects of salicylate in varying dosage appear to be chiefly related to the correspondingly varied concentrations of free salicylate in the tubular urine (8). This relationship, illustrated in Figure 5, is represented by a discontinuous curve. Low ranges of urinary excretion of free salicylate are associated with decreased C_{urate}/C_{inulin} ratios, which at first decline further as urinary excretion of free salicylate slowly increases. The point of minimum inflection is formed by the intersection with another, sharply rising curve which represents increasing C_{urate}/C_{inulin} ratios, to markedly uricosuric levels, as urinary excretion of free salicylate further increases. The inexplicable apparent loss of drug potency at intermediate dosage levels clearly is the resultant of opposing suppressive and augmentative forces, in such relative proportions as virtually to nullify each other, and thus yield an insignificant net effect.

These results can be reconciled with the conventional concept of renal regulation of urate excretion only with some difficulty. The uricosuric effect of large doses of salicylate, associated with markedly increased urate/inulin clearance ratios, is readily understandable as due to inhibition of tubular reabsorption of urate, as in the case of other potent uricosuric agents (38–42). The suppressive effect of small doses of salicylate, however, requires the rather awkward assumption of *stimulation* of tubular reabsorption of urate by the same drug, and without any change in filtered urate load. A similar explanation presumably would apply to urate retention caused by phenylbutazone in low dosage (7) and by pyrazinamide (43) and lactate (44). The apparent impotency of intermediate doses of salicylate poses a dilemma. The single tubular transport system for urate would seem to be simultaneously stimulated and suppressed by the same drug in the same dosage.

The alternative concept of renal regulation of urate excretion, which postulates a tubular excretory as well as reabsorptive transport mechanism, supposes the suppressive effect of small doses of salicylate (and other agents with like action) to be due to inhibition of tubular secretion of urate (7, 8, 32, 33).[2] When the concentration of free salicylate in the tubular urine is sufficiently increased, it is assumed that inhibition of the reabsorptive mechanism also occurs. In the intermediate range of urinary excretion free salicylate, inhibition of urate secretion is marked whereas inhibition of urate reabsorption is so slight that the net effect is virtually to cancel out the effect of either action alone, the net result on the ex-

[2] The term "excretion" as employed in this discussion is used in a general sense, with no inferences as to whether excretion is "active," "passive" or both. "Secretion" here implies "active" tubular transfer.

cretion of urate being negligible. High concentrations of free salicylate in the tubular urine are considered markedly to inhibit both tubular reabsorption and secretion of urate (7, 8, 32, 33). The net effect of such marked dual inhibition would be expected to be uricosuric, since the quantity of urate normally reabsorbed from the glomerular filtrate in man is 10 to 20 or more times greater than the quantity eliminated in the urine.

The nature of the inhibition of the reabsorptive (and secretory) processes of tubular transfer of urate, by salicylate and the other drugs in question, must remain a matter of speculation for the present. Competition for some common component of the reabsorptive and secretory tubular transfer mechanisms may occur.

The interacting influences of salicylate and probenecid, when jointly administered, on the tubular regulation of urate excretion

The inhibition of probenecid uricosuria by small doses of salicylate poses a further dilemma for the conventional concept of the renal regulation of urate excretion, since here again there would seem to be simultaneous inhibition and stimulation of a single tubular transfer system, in this instance by two different drugs. A similar difficulty arises in connection with the inhibition of probenecid uricosuria by other drugs which, when given alone, cause marked retention of urate (for example, lactate) (44).

The explanation alternatively offered postulates that the inhibition of tubular reabsorption of urate produced by probenecid is partially counterbalanced by simultaneous inhibition of tubular secretion of urate by salicylate in low dosage (33). A similar dual mechanism is presumed to underlie the inhibitory effect of small doses of salicylate on the enhanced urate excretion produced by other potent uricosuric agents, such as zoxazolamine (42) and the phenylbutazone analogs, G-25671 and G-28315 (45, 46). It is interesting to note that salicylate, which has no effect on tubular secretion of penicillin, in no way influences the inhibition of penicillin secretion by probenecid (47).

It should be pointed out, however, that the observed counterbalance of probenecid uricosuria by small doses of salicylate is greater than anticipated by the postulated inhibition of tubular secretion of urate, since the quantity of urate secreted apparently is small in relation to that reabsorbed. Other factors, as yet obscure, must therefore be presumed also to be operative. Another discrepancy is the failure to observe an additive effect if salicylate in large (uricosuric) dosage is given when probenecid uricosuria is already produced, or if probenecid is administered when salicylate uricosuria already obtains (9, 48, 49). Such an additive effect is in fact noted when zoxazolamine and G-28315 are administered jointly, or with probenecid (42, 50).[3] However, in the case of salicylate uricosuria, probenecid exerts a moderate depressant effect, associated with a decline in the free salicylate concentration in the tubular urine. This response seems to be related to the inhibitory effect, already referred to (11, 19), of probenecid on the tubular secretion of salicylate.

SUMMARY

1. In 44 experiments in 23 gouty subjects the administration of acetylsalicylic acid in daily oral doses of 1.0, 2.0, 3.0 and 5.2 Gm. per day produced mean changes in 24 hour urinary urate excretion of −21, −14, +16 and +37 per cent, respectively. When 5.2 Gm. per day sodium bicarbonate was added to 5.2 Gm. per day salicylate dosage, the mean 24 hour urinary urate excretion rose to +86 per cent. Corresponding inverse changes were observed in serum urate levels.

2. Renal clearance studies in eight subjects slowly infused with sodium salicylate revealed that low levels of plasma salicylate (of the order of 1.5 to 10 mg. per cent) and of urinary excretion of free salicylate (of the order of 0.05 to 0.5 mg. per minute) were usually associated with a distinct fall in C_{urate}/C_{inulin} below premedication ratios. High levels of plasma salicylate (> 10 or 12 mg. per cent) and of urinary excretion of free salicylate (0.5 to 1.0 or more mg. per minute) were usually associated with a pronounced rise in C_{urate}/C_{inulin} above premedication ratios.

[3] Additive uricosuric effects are obtained with concurrent administration of uricosuric agents which do not cause urate retention, when given in low dosage, as in the case of zoxazolamine and G-28315. Probenecid in very low dosage may cause slight retention of urate [maximum observed, 27 per cent (7)] but this is not a constant finding.

Plasma salicylate levels and urinary excretion of free salicylate within a zone intermediate between those causing distinct urate retention and uricosuria were usually associated with little or no significant change from the premedication C_{urate}/C_{inulin} ratios.

3. Dissociation of plasma and urinary salicylate levels, by rendering the urine distinctly acid (ammonium chloride administration) or alkaline (sodium bicarbonate), indicated that the paradoxical effects of salicylate on C_{urate}/C_{inulin} were determined at the tubular level, largely by the concentration of free salicylate in the tubular urine. In subjects with distinctly acid urine, urinary excretion of free salicylate remained relatively low even after large sodium salicylate infusions, the plasma salicylate rose inordinately, the rise in C_{urate}/C_{inulin} was delayed and less pronounced than ordinarily. In subjects with urine made alkaline with sodium bicarbonate, urinary excretion of free salicylate increased sharply and the rise in C_{urate}/C_{inulin} was considerably greater than that produced by salicylate alone. In subjects with urine made alkaline with acetazolamide, urinary excretion of free salicylate again rose sharply but the increased C_{urate}/C_{inulin} produced by salicylate alone was somewhat depressed.

4. It is suggested that the paradoxical effects of salicylate on urinary urate excretion can be reasonably explained on the assumption that the filtered urate load in man is virtually completely reabsorbed by the tubules and that the urate in the urine, contrary to the conventional view, is derived largely by tubular secretion. It is postulated that low concentrations of free salicylate in the tubular urine depress tubular secretion of urate. Intermediate concentrations of free salicylate, it is suggested, depress tubular secretion, and also reabsorption of urate to a degree sufficient to give little or no net change in urate excretion. High concentrations of free salicylate in the tubular urine are believed to suppress tubular reabsorption of urate markedly (as well as tubular secretion), the net effect being uricosuric.

5. It is further suggested that probenecid uricosuria, which is produced by inhibition of tubular reabsorption of urate, is counteracted by small doses of salicylate in part because of concurrent suppression of tubular secretion of urate.

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A CORRELATION OF CLINICAL AND HEMODYNAMIC STUDIES IN PATIENTS WITH HYPERTHYROIDISM WITH AND WITHOUT CONGESTIVE HEART FAILURE *

By JOHN S. GRAETTINGER, JOSEPH J. MUENSTER,† LOUIS A. SELVERSTONE ‡ AND JAMES A. CAMPBELL

(From the Cardio-Respiratory Division, Department of Medicine, Presbyterian Hospital, and Department of Medicine, University of Illinois College of Medicine, Chicago, Ill.)

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The occurrence of congestive heart failure during the course of hyperthyroidism has frequently been observed, as has its amelioration after reestablishment of the euthyroid state. The majority of patients in whom congestive failure has appeared have been believed to have myocardial disease of other etiology (1, 2), but the probability that the circulatory load imposed by hyperthyroidism can cause even the otherwise healthy heart to fail has also been suggested (3–5). During investigation of the hemodynamic consequences of the endogenous circulatory loads imposed by various disorders, we have obtained clinical and physiologic data in 20 patients with hyperthyroidism, seven of whom were or had been in congestive heart failure. At the time of study we were unable to determine whether the congestive failure was principally attributable to myocardial damage antedating thyrotoxicosis, to a possibly direct toxic action of thyroxin on the myocardium, or might be due to the circulatory load imposed by this disease overcoming the reserve of an otherwise healthy myocardium. Evaluation of these patients after they had become euthyroid disclosed that only five had residual heart disease. The measurements made in these patients with congestive failure differed significantly from those obtained in hyperthyroid patients not in failure; furthermore, the patients in whom congestive failure had occurred could be subdivided into two significantly different groups on the basis of the hemodynamic data. It is our purpose in the present paper to contrast the physiologic aberrations in hyperthyroidism uncomplicated by congestive failure with the abnormalities found in patients in whom hyperthyroidism and congestive heart failure coexist with or without antecedent heart disease.

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‡ Present address: New England Center Hospital, Boston, Mass.

CLINICAL MATERIAL

Hemodynamic data obtained in 21 studies performed in 20 patients with hyperthyroidism form the basis of this report. Each patient had had symptoms and signs of thyrotoxicosis for more than three months. The patients were divided into two major groups shown in Table I on the basis of the presence or absence of congestive heart failure on clinical examination during the course of their illness prior to physiologic study.

Group I consists of 14 patients who had not manifested congestive failure. Eight of these 14 patients had no evidence of cardiorespiratory disease and comprise Group IA. The other six patients had evidence of heart disease and comprise Group IB. The average age of the eight Group IA patients was 34, with a range of 24 to 52, years. Exertional fatigue and breathlessness were noted by seven of these eight patients but resulted in slight or no limitation in exercise tolerance. Their physical examinations were unremarkable except for typical evidence of hyperthyroidism. Grade I or II upper left sternal border systolic murmurs were noted in three, and Grade I apical systolic murmurs in two, of these patients. Teleroentgenograms in these patients revealed normal cardiac size and contour in six and slight prominence of the right ventricular outflow tract in two (E. M., M. H.). Electrocardiograms in five showed sinus tachycardia and in two normal sinus rhythm. Increased QRS voltage was noted in the unipolar precordial leads of six of the eight (maximum S wave plus maximum R wave over 40 mm.). Total blood volume estimated with I^{131} and related to presenting body weight was normal in each of the three in whom this measurement was made (E. M., G. C., M. H.).

The other six patients with thyrotoxicosis uncomplicated by congestive failure but with definite evidence of heart disease are designated Group IB. The average age of these patients was 47 years, with a range of 28 to 56. Four had hypertensive heart disease (J. J., M. R., F. S., M. He.) with long-standing diastolic hypertension, ab-

normal retinal arterioles and left ventricular hypertrophy by electrocardiographic and roentgenographic examination. Another patient (L. D.), aged 37, had moderate cardiac enlargement with electrocardiographic evidence of increased QRS voltage and changes suggestive of diffuse injury and ischemia which evolved to normal during the initial month of therapy for her hyperthyroidism. The final patient (L. P.) had rheumatic heart disease with mitral regurgitation; her electrocardiogram was compatible with left atrial and ventricular hypertrophy as were her roentgenographic studies.

Despite the evidence of hyperthyroidism and heart disease in each patient no evidence of congestive failure was found. Although abnormalities of the cardiac silhouette were present in teleroentgenograms in each of the patients, in no instance was evidence of pulmonary congestion noted. Electrocardiograms in five of the six disclosed a sinus mechanism and atrial fibrillation was present in one (F. S.) at the time of study. Transient atrial fibrillation during hospitalization had occurred, however, in three of the other five (J. J., M. R., L. P.). Arm to lung circulation times ranged from 11 to 17 seconds in the four patients in whom this determination was made. Total blood volumes were within normal limits in two patients (F. S., L. D.) and increased by 30 per cent in one (L. P.). One of the patients (M. R.) had a significant anemia.

Group II consists of seven other patients with hyperthyroidism, in each of whom congestive heart failure had occurred during the course of the disease. They are separated into two groups by the levels of their cardiac outputs.

Group IIA consists of four patients with normal cardiac outputs:

M. He. was a 68 year old female with hyperthyroidism and hypertensive heart disease who had been studied previously (Group IB). She had not become euthyroid. A year after the first study she entered the hospital in moderately severe congestive failure which had cleared by the time of her second study.

N. S. was a 42 year old female who presented with evidence of rheumatic heart disease with mitral and aortic regurgitation, atrial fibrillation and congestive failure. Following physiologic studies she responded well to antithyroid medication and her congestive failure cleared.

S. S.[1] was a 36 year old male who rapidly developed severe congestive failure in the week prior to admission. On examination he presented marked pulmonary and peripheral edema, a Grade III apical holosystolic murmur, atrial fibrillation, and in teleroentgenograms had diffuse cardiac enlargement with pulmonary congestion. He lost 40 pounds in a week with improvement in the signs of congestive failure, but cardiac enlargement and pulmonary congestion persisted in his chest films at the time of study. It was felt that he probably had antecedent rheumatic heart disease. He was treated with I^{131}. Two years after study he was entirely well; the cardiac examination and teleroentgenograms were normal and electrocardiograms revealed that he had reverted to normal sinus rhythm.

H. K. was a 55 year old female who presented evidence of hypertensive cardiovascular disease with congestive failure in addition to hyperthyroidism. Atrial fibrillation was present. Teleroentgenograms disclosed diffuse cardiac enlargement and congestion of the lung fields. Six months later following antithyroid therapy her thyrotoxicosis and congestive failure were much improved.

Group IIB consists of three patients who had unusually high cardiac outputs:

G. F.[2] was a 38 year old male in whom a diagnosis of hyperthyroidism was made two years prior to study. Various antithyroid drugs had been given until a few weeks prior to admission. In the five months prior to observation, dyspnea and edema appeared and progressed despite digitalis and diuretic therapy. Examination revealed severe congestive failure. Teleroentgenograms showed diffuse cardiac enlargement with pulmonary congestion; an electrocardiogram showed atrial fibrillation at a rate of 124 without other abnormality. Physiologic studies were performed eight days following admission at which time his hemoglobin had fallen to 8.3 Gm. Two and one-half years later his functional capacity was Class I and a teleroentgenogram showed normal heart size and contour; atrial fibrillation persisted without other electrocardiographic abnormality. His hemoglobin was normal.

P. R. was a 49 year old female who presented with severe congestive failure. An electrocardiogram showed atrial fibrillation at a rate of 245 with runs of ventricular tachycardia. Blood volume was 35 per cent above predicted. A teleroentgenogram was interpreted as normal. She was treated with oxygen, morphine, Lugol's solution and propylthiouracil and rapidly digitalized. Physiologic studies were performed 19 days after admission, at which time her neck veins were not distended and her lung fields were clear. Serial electrocardiograms showed progressive decrease in ventricular rate with persistence of fibrillation and nonspecifically abnormal ST-T deflections. She was readmitted nine months later because of recurrence of dyspnea, orthopnea and edema. Her heart was enlarged; atrial fibrillation and 3+ edema were present. Her basal metabolic rate was +47 per cent. A thyroidectomy was performed followed by remission of symptoms and signs of heart disease despite the persistence of atrial fibrillation for the six month follow-up period available.

I. J. was a 28 year old female who presented with a history of hyperthyroidism of 22 months' duration and congestive failure of six months' duration with known hypertensive heart disease. An electrocardiogram showed normal sinus rhythm and was compatible with left ventricular hypertrophy. After physiologic studies she

[1] This patient was studied through the courtesy of Dr. Lyle A. Baker, Chief of Medicine, Veterans Administration Hospital, Hines, Ill.

[2] This patient was studied through the courtesy of Dr. Lyle A. Baker, Chief of Medicine, Veterans Administration Hospital, Hines, Ill.

TABLE I

Hemodynamic data in patients with thyrotoxicosis

Patient	Age	Sex	Surface area	Hemoglobin	Basal metabolic rate	Oxygen consumption R	Oxygen consumption E	Cardiac index R	Cardiac index E	Cardiac output (*L./min.* × *100*) / Oxygen consumption (*ml.*) R	Cardiac output (*L./min.* × *100*) / Oxygen consumption (*ml.*) E	Stroke index R	Stroke index E	Heart rate R	Heart rate E
			M.²	*Gm.*		*ml./min./M.²*		*L./min./M.²*				*ml./beat/ M.²*		*beats/ minute*	
Group I---No congestive failure															
A. No heart disease (8)															
J. A.	27	F	1.75	11.6	+51 +42	174	283	4.54	5.42	2.61	1.92	44	48	103	112
S. P.	57	F	1.26	13.2	+34* +46	168	298	5.20	6.42	3.10	2.15	41	46	128	140
G. C.	24	F	1.63	12.0	+49 +49	187	272	6.64	9.53	3.55	3.50	63	75	104	127
E. M.	29	F	1.62	12.5	+55* +53	194	425	6.27	8.49	3.23	2.00	48	57	132	150
M. H.	30	F	1.50	10.7	+23* +62	201	267	5.95	5.91	2.96	2.21	60	57	99	103
R. E.	52	M	1.79	12.8	+60* +68	211	344	5.55	8.00	2.63	2.33	50	67	110	120
B. R.	24	M	1.72	12.3	+85 +81	252	395	7.44	14.26	2.95	3.61	66	116	113	123
E. A.	32	F	1.66	12.7	+33 +83	225	381	6.25	7.76	2.78	2.04	73	75	86	103
Mean	34		1.62	12.2	+61	202	333†	5.98	8.22†	2.98	2.47†	56	68	109	122†
Standard error						10	22	0.32	0.99	0.11	0.24	4	8	5	6
B. Heart disease (6)															
J. J.§	56	M	1.73	13.6	+38 +31	164	347	2.90	5.47	1.77	1.58	40	66	71‖	83
M. R.	46	F	1.90	9.5	+53* +41	172	249	4.77	5.13	2.77	2.06	49	51	98‖	100
F. S.	46	M	1.74	14.7	+36* +41	183	312	2.84	3.68	1.55	1.18	24	23	120¶	160
L. D.	37	F	1.62	11.8	+35 +47	181	348	4.58	6.00	2.53	1.72	52	63	87	95
L. P.	28	F	1.51	14.1	+74* +75	205	411	5.08	8.43	2.48	2.05	53	70	95‖	120
M. He.	66	F	1.43	13.3	+45 +86	208	403	6.31	6.40	3.03	1.59	55	49	116	132
Mean	47		1.66	12.8	+54	185	345†	4.41	5.85†	2.36	1.70†	46	54	98	115†
Standard error						7	25	0.55	0.64	0.27	0.14	5	7	7	11
Group II—Congestive failure															
A. Normal cardiac output (4)															
M. He.	68	F	1.46	13.1	+26* +21	147	207	2.51	2.89	1.71	1.40	28	27	89	107
N. S.	42	F	1.78	9.6	+29* +49	175	371	4.45	4.91	2.54	1.32	45	35	100¶	140
H. K.	55	F	1.65	12.3	+45* +96	225	262	3.31	3.67	1.47	1.40	30	31	110¶	119
S. S.§	36	M	1.24	13.5	+54* +54	206	263	3.12	3.83	1.51	1.46	25	31	125¶	125
Mean	50		1.53	12.1	+55	188	276†	3.35	3.83	1.81	1.40	32	31	106	123
Standard error						17	34	0.41	0.42	0.25	0.03	4	2	8	7
B. High cardiac output (3)															
G. F.§	38	M	1.94	8.3	+25* +24	166	273	8.26	7.10	4.98	2.60	66	45	124¶	156
P. R.§	49	F	1.52	11.6	+71 +70	205	249	8.50	6.68	4.15	2.68	65	39	129¶	173
I. J.	28	F	1.96	11.2	+25 +110	259	297	10.04	8.91	3.88	3.00	90	70	111	127
Mean	38		1.81	10.3	+68	210	273	8.93	7.56†	4.34	2.76	73	51‡	121	152
Standard error						27	14	0.56	0.68	0.33	0.12	8	9	5	13
Normals (6)															
Mean	36		1.71	13.4	− 5	127	222†	2.98	4.26†	2.33	1.92	39	49	76	87†
Standard error						6	13	0.25	0.42	0.36	0.14	3	5	3	2

TABLE I—*Continued*

A–V oxygen difference		Right atrial pressure		Mean pulmonary artery pressure		Brachial artery pressure				Total pulmonary resistance		Total systemic resistance		Left ventricular work index	
						R		E							
R	E	R	E	R	E	S/D	M	S/D	M	R	E	R	E	R	E
ml./100 ml.		*mm. Hg*		*mm. Hg*		*mm. Hg*				*dynes-sec.-cm.$^{-5}$*		*dynes-sec.-cm.$^{-5}$*		*Kg. M./min./M.^{2}*	
3.84	5.22	12	12	19		117/70	91	134/74	98	191		916	826	5.62	7.21
3.23	4.65	5	7	23	24	133/64	91	143/72	103	280	237	1,190	1,018	6.44	8.98
2.82	2.85	5	5	19		126/69	87	127/68	89	140		643	458	7.87	11.53
3.08	5.00	4	5	17	25	125/72	84	134/71	88	134	145	661	512	7.16	10.19
3.38	4.52	8	5	21	21	119/68	84	129/72	87	188	189	752	784	6.80	6.99
3.80	4.30	10	14			112/72	85	126/74	91			520	508	6.41	9.90
3.38	2.77		1			114/59	80	151/75	112			493	422	8.11	18.76
3.60	4.91	3	1	16	20	97/60	73	137/80	103	123	124	562	757	6.20	10.87
3.39	4.28†	7	6	19	23	118/67	84	135†/73†	96‡	184	183	717	660‡	6.83	10.55‡
0.12	0.34	1	2	1	1	4–4	2	5–3	3	27	33	83	76	0.30	1.30
5.66	6.34	−3	1	12	12	154/72	101	178/82	158	191	101	1,608	1,334	4.02	11.75
3.6‡	4.85	9	15	27	35	140/68	100	166/92	127	238	287	883	1,041	6.48	8.67
6.44	8.49	11	13	29	42	167/104	125	204/131	155	469	524	2,022	1,459	7.14	10.29
3.95	5.80	6	13			133/73	99	160/89	119			1,066	978	6.17	9.71
4.04	4.88	2	4	20	23	104/64	87	110/69	86	208	144	907	540	6.01	9.86
3.30	6.36			RV 34/0	RV 28/0	146/68	112	187/72	128			991	1,118	9.62	11.15
4.50	6.12†	5	11†	22	28	141/75	104	168†/89†	129†	277	264	1,246	1,078	6.57	10.24†
0.50	0.55	3	3	4	7	9–6	5	13–9	11	65	95	189	131	0.74	0.45
5.83	7.16	2	4	12		149/64	95	162/70	107	262		2,070	2,030	5.26	6.21
3.94	7.56	24	18	19	24	118/65	87	135/70	93	192	242	878	850	5.22	6.29
6.81	7.14	13	18	39	53	169/75	116	185/90	126	571	699	1,698	1,662	3.52	4.32
6.59	6.86	10		25	33	122/63	83	129/64	83	516	555	1,714	1,396	3.25	4.21
5.79	7.18	12	13	24	37‡	140/67	95	153†/74	102	385	499	1,590	1,484	4.31	5.26
0.65	0.14	4	5	6	9	12–3	7	13–6	3	93	186	253	248	0.54	0.59
2.01	3.85	18	26	35	39	134/52	79			175	226	394		8.87	
2.42	3.73	7	7			165/91	119	167/94	121			737	941	13.73	11.04
2.58	3.34	8	12			120/75	95					386		13.42	
2.33	3.64	11	15			140/73	98					506		12.01	
0.17	0.15	4	6			13–11	12					116		1.55	
4.41	5.36	4	3	15	18	134/76	100	135/78	103	242	207	1,636	1,130†	3.58	6.16
0.23	0.14	1	1	5	6	8–3	5	8–4	2	30	35	118	123	0.33	0.81

* PBI > 12 μg. per 100 ml. or RAI uptake > 60 per cent.
† $p < 0.02$.
‡ $p < 0.05$.
§ On digitalis at time of study.
‖ Transient atrial fibrillation during hospitalization.
¶ Atrial fibrillation at time of study.

was treated and became euthyroid. Nine months later she was admitted for thyroidectomy and was asymptomatic. Examination revealed blood pressures ranging from 152/100 to 170/110 and evidence of hypertensive heart disease without congestive failure.

Basal metabolic rates of each patient are shown in Table I, with the first value being the lowest determination recorded in the two weeks prior to physiologic study and the second value being the measurement made at the time of study. The results of serum protein-bound iodine and/or thyroidal uptake of I^{131} determinations in these patients are indicated by an asterisk.

Methods

The studies were performed in an air-conditioned room on patients in the postabsorptive state, usually after a sedative dose of sodium allyl barbiturate. Details of the methods used in this laboratory have been reported previously (6). Cardiac output was determined at rest and during the fourth minute of exercise performed supine on a stationary bicycle. Total systemic and pulmonary resistances were calculated as follows:

$$\text{total systemic resistance (dynes-sec-cm.}^{-5}) = \frac{BA_M \times 1{,}332}{C.O.}$$

and

$$\text{total pulmonary resistance (dynes-sec-cm.}^{-5}) = \frac{PA_M \times 1{,}332}{C.O.}$$

where BA_M = brachial arterial mean pressure (mm. Hg), PA_M = pulmonary arterial mean pressure (mm. Hg) and C.O. = cardiac output (ml. per second). "Left ventricular work index" was approximated, in the absence of pulmonary capillary wedge pressures, by the calculation:

$$\text{left ventricular work (Kg. M. per minute per M.}^2) = \frac{CI \times BA_M \times 1.36}{1{,}000}.$$

Results

The data obtained in these patients are shown in Table I together with values obtained in healthy subjects studied in our laboratory with which they are compared.

Group IA—Thyrotoxicosis without heart disease or congestive failure

The mean cardiac output index of 5.98 ± S.D. 0.89 L. per minute per M.^{2} in these eight patients was abnormally elevated ($p < 0.001$); the ratio of the cardiac index to the level of oxygen consumption index of 2.98 ± S.D. 0.31 L. per 100 ml. was also significantly greater than in the healthy subjects ($p < 0.01$). During exercise the mean increase of the cardiac output was significant ($p < 0.02$). In two patients (G. C., B. R.) a marked hyper-response of output during exercise was observed. In one patient (M. H.), whose metabolic rate at the time of study was considerably more elevated than on the ward, little further increase in oxygen consumption and no change in the cardiac output occurred during exercise.

The increased resting output in these patients was occasioned both by a greater stroke volume index ($p < 0.02$) and heart rate ($p < 0.001$) than normal. During exercise a significant increase in heart rate occurred and the stroke volume increased from 4 to 50 ml. per beat per M.^{2} in six of the eight patients; the mean increase was not significant. Atrial fibrillation was not observed.

The arteriovenous oxygen difference of 3.39 ± S.D. 0.35 ml. per 100 ml. at rest was significantly lower than normal ($p < 0.01$). During exercise, the mean arteriovenous oxygen difference widened normally; in the two patients in whom marked hyper-response of the cardiac output occurred, no change or actual decrease in oxygen extraction occurred. Arterial oxygen saturation was normal in each patient.

Mean right atrial and mean pulmonary arterial pressures for the group were high normal at rest and did not increase with exercise although a significant rise ($p < 0.05$) in pulmonary arterial systolic pressure occurred. Two patients (J. A., R. E.) had definite atrial hypertension at rest. Calculated total pulmonary resistances were low normal and did not change with exercise.

At rest total systemic resistance was lower than normal ($p < 0.001$). During exercise, despite a significant rise in systolic, diastolic and mean ($p < 0.05$) arterial pressures, a further significant decrease in systemic resistance occurred ($p < 0.05$). The left ventricular work index was greater than normal ($p < 0.001$) and increased significantly with exercise. The level of left ventricular work at rest in this group (6.83 ± 0.85 Kg. M. per minute per M.^{2}) equaled that of the normals during exercise (6.16 ± 1.99 Kg. M. per minute per M.^{2}).

Group IB—Thyrotoxicosis with antecedent heart disease but without congestive failure

The resting mean cardiac index of 4.41 ± S.D. 1.33 L. per minute per M.^{2} in these pa-

tients was greater than normal ($p < 0.05$) but the ratio of cardiac output to oxygen consumption did not differ from normal in contrast to Group IA. The exercise response of cardiac output for the group did not differ from normal or Group IA. The ratio of cardiac output to oxygen consumption was lower at rest ($p < 0.05$) and during exercise ($p < 0.05$) than in Group IA. Two patients (J. J., F. S.) had relatively low outputs at rest. One (J. J.) was the only one in the group who had been digitalized; he showed a normal increase in output with exercise. The other patient, F. S., had a relatively low output during exercise as well, despite the expected increase in oxygen consumption. He was the only patient in this group who presented atrial fibrillation at the time of study. Another patient, M. He., failed to show an increase in output with exercise despite a marked increase in oxygen consumption; she subsequently developed congestive failure (*vide infra,* Group IIA). The mean stroke volume index for the group was not significantly elevated at rest nor did it increase with exercise. The mean heart rate was elevated at rest ($p < 0.05$) and increased with exercise. In addition to the one patient with atrial fibrillation at the time of study three other patients (J. J., M. R., L. P.) had transient fibrillation during hospitalization prior to study. The mean arteriovenous oxygen difference was greater than in Group IA ($p < 0.05$) and did not differ from normal. With exercise it increased considerably to a high normal level which was greater than in Group IA ($p < 0.02$). Mean right atrial pressure at rest was normal but increased significantly during exercise in contrast to Group IA. Patient F. S. had atrial hypertension. Mean pulmonary arterial pressure was in the high normal range at rest and during exercise. Slight pulmonary hypertension developed during exercise in the patient with atrial fibrillation (F. S). With one exception (L. P.) the mean brachial artery pressure both at rest and during exercise was higher in each patient in this group than in any patient in Group IA, which was expected since four of the six had hypertensive heart disease. Mean brachial artery pressure for the group was not higher than in the normal subjects, however, and systemic resistance at the time of study was in the normal range. With exercise mean brachial artery pressure rose and systemic resistance did not change. Pulmonary resistance did not differ from normal. The left ventricular work index was elevated at rest ($p < 0.01$) and rose with exercise.

II. Thyrotoxicosis complicated by congestive failure

Group IIA. In these four patients the mean cardiac index at rest was not different from normal. During exercise no significant change in output occurred in contrast to normal subjects. The ratio of cardiac output to oxygen consumption at rest and during exercise was lower in each patient than in any patient in Group IA. The absolute level of cardiac index during exercise for this small group was lower than normal ($p < 0.05$). The stroke volume index was in the normal range and did not increase during exercise. The heart rate was elevated at rest and increased in three of the four during exercise. The mean arteriovenous oxygen difference for the group was abnormally widened at rest and exercise when compared with normal ($p < 0.05$). Mean atrial pressure was elevated at rest and exercise compared with normals ($p < 0.02$). Patient M. He., who was not clinically in failure at the time of study, had a normal atrial pressure. A significant pulmonary hypertension ($p < 0.02$) occurred with exercise. Pulmonary resistance was high normal at rest and exercise. Although a significant increase in left ventricular work occurred with exercise, the absolute level during exercise was not different from that in Group IA or IB at rest.

Group IIB. The hemodynamic data obtained in these three patients differed strikingly from the other patients studied. The mean cardiac index of $8.93 \pm$ S.D. 0.97 L. per minute per $M.^2$ calculated for this small group was higher than in the normals ($p < 0.001$), Group IA ($p < 0.001$) or in Group IIA ($p < 0.001$), and the mean ratio of cardiac output to oxygen consumption was significantly greater than in Group IA ($p < 0.001$). With exercise a marked drop in cardiac index occurred in each patient in this group in contrast to all of the other patients. The mean stroke index for the group was higher than normal ($p < 0.01$) and decreased with exercise. The mean heart rate for the group was elevated at rest ($p <$

0.001) and increased in each during exercise; two of these three patients had atrial fibrillation. The mean arteriovenous difference for the group was quite narrowed both at rest and exercise ($p < 0.01$). Mean atrial pressures were elevated at rest and became yet more elevated during exercise in two of the three; normal values were obtained in P. R. who was not in failure clinically at the time of study. Pulmonary arterial pressure was elevated in the one patient in whom it was measured. Systemic resistance was markedly reduced in each patient and left ventricular work was increased at rest to a level higher than the exercise level in all but one (B. R.) of the patients in the entire series.

Discussion

The circulatory responses to thyrotoxicosis observed in the seven patients without heart disease or congestive failure (Group IA) at rest were, in general, similar to those described by others (7–16). The responses of the cardiac output and systemic resistance to mild exercise in these patients were qualitatively similar to healthy persons and the increased blood flow associated with thyrotoxicosis both at rest and during exercise occurred without significant change in right atrial or pulmonary arterial pressures. These data strongly suggest myocardial competence and have been used as a basis for comparison with the other groups.

Since hyperthyroid patients both without congestive failure (Group IA) and some of those with congestive failure (Group IIB) had narrowed arteriovenous differences and thus greater than normal ratios of cardiac output to oxygen consumption, the commonly used relationship of cardiac output to oxygen consumption as a measure of cardiac competence was not possible. Although any of these hyperthyroid individuals might not have been basal at the time of the "rest" study and hence factors tending to elevate the cardiac output out of proportion to oxygen requirement might have been operative, the operation of such factors was largely overcome by the exogenous load of exercise. The exercise cardiac output and arteriovenous difference data clearly separate the four groups of patients and the arteriovenous difference remained less than the normal during exercise in the second group of patients who had had congestive failure (Group IIB) as well as in the patients without failure (Group IA). We assume, therefore, that regardless of the absolute level of cardiac output its change with exercise was the best indicator of cardiac competence for the evaluation of these patients.

The patients in Group IB were initially classified as not having myocardial inadequacy despite the presence of heart disease because of the absence of congestive failure. This clinical impression was confirmed by the qualitatively normal cardiac output response to exercise. Although the cardiac output was elevated in this group with respect to normal, the ratio of the cardiac output to the level of oxygen consumption was less than in the thyrotoxic patients without heart disease. More elaborate measurements of oxygen demand and output response relationship (17) in patients of this kind would be of particular interest, since the significant rise in atrial pressure during exercise in this group also suggests some degree of impairment in myocardial function.

The physiologic data in the patients who had had clinical congestive failure differed markedly from those obtained in the patients without failure. In four of the patients (Group IIA) the cardiac output was in the normal range at rest but was quite inadequate with respect to the elevated oxygen consumption as reflected in the low ratio of cardiac output to oxygen consumption and the widened arteriovenous oxygen difference. This group therefore presented relatively "low output" failure when compared with the patients with thyrotoxicosis without congestive failure (Group IA). When compared with seven euthyroid patients with congestive failure on the basis of primary myocardial disease (6) in whom the cardiac output averaged 1.93 ± S.D. 0.31 L. per minute per $M.^2$, however, these patients had relatively "high output" failure. The elevation of right atrial pressure at rest and of atrial and pulmonary arterial pressure during exercise and the failure of the cardiac output to rise or of systemic resistance to decrease with exercise found in this group were entirely similar to the findings in euthyroid patients with heart failure due to myocardial disease. Three of the four patients in this group had definite heart disease antedating the onset of thyrotoxicosis.

The findings in the fourth patient, S. S., are of

particular interest. At the time of study, despite his young age and lack of definitive evidence of rheumatic heart disease, he was considered as probably having heart disease antedating his thyrotoxicosis on the basis of the clinical and hemodynamic data. Over the course of the two years following study as he returned to full health and eventually had an entirely normal exercise tolerance and cardiovascular examination, our original impression became untenable and he was categorized as a patient in whom the hyperthyroid state had transiently produced myocardial inadequacy and congestive heart failure.

In the other group of patients who had presented congestive failure clinically (Group IIB) the decrease in cardiac output with exercise in the presence of elevation of the right atrial pressure in two of the three and of pulmonary arterial pressure in the one in whom these data were obtained were also similar to euthyroid patients with congestive failure. The striking finding in each of these patients, however, was a cardiac output and cardiac output to oxygen consumption ratio which was not only considerably greater than normal but also greater than in any patient with thyrotoxicosis not in failure (Group IA). The first of these patients had a moderate anemia in addition to hyperthyroidism at the time of study which contributed to his circulatory load. We have found, however, that a considerable hyperresponse of cardiac output characteristically occurs during exercise in patients with hemoglobins of 8 Gm. (18) in contrast to the fall in cardiac output observed in this patient. He has since totally recovered, and resembles the young patient described in 1929 by Burwell, Smith and Neighbors (19) who presented a combination of thyrotoxicosis and pernicious anemia, in whom the cardiac output was far above that expected on the basis of metabolic demand, and who also apparently had no antecedent or residual heart disease after treatment of his anemia and thyrotoxicosis. The second patient in this group was older, had a questionable history of angina pectoris, had had severe cardiac irritability with runs of ventricular tachycardia on entry and remained in clinical congestive failure for nine months until she became euthyroid following thyroidectomy, at which time she presented no signs of heart disease. The third patient had mild hypertensive heart disease without clinically apparent decrease in myocardial reserve until the development of hyperthyroidism and subsequent congestive failure. Thus, in two of these three patients no antecedent or residual heart disease was evident; in the third, only mild hypertensive heart disease was present before and after thyrotoxicosis.

Elucidation of the genesis of the clinical syndrome of congestive failure in these patients requires a consideration of the cardiovascular stresses imposed by thyrotoxicosis and the mechanisms by which they may cause myocardial incompetence.

Despite the absence of characteristic cardiac morphologic abnormalities in patients who have succumbed with thyrotoxicosis and heart failure (5, 20, 21), a direct alteration of myocardial function by thyroxin has been demonstrated in both clinical and experimental hyperthyroidism (15, 22–30). Impairment of myocardial energy production due to altered enzyme function may have been a factor involved in the failure of an adequate cardiac output response to exercise (31–35). The action of thyroxin on myocardial function may well be mediated by sensitization of the myocardium to sympathetic nerve stimulation and to sympathomimetic amines (36–40). An increased concentration of catechol amines has also been demonstrated in the myocardia of thyrotoxic dogs (41). The hyper-response of cardiac output to exercise (G. C., B. R.) and the occurrence of paroxysmal ventricular tachycardia in Patient P. R. may well be attributable to this phenomenon (42, 43). Furthermore the possibility that myocardial hypoxia and hence impaired function may be caused by excessive catechol amine concentrations has also been mentioned (41).

In addition to a possible direct impairment of myocardial metabolism, thyrotoxicosis frequently is associated with the occurrence of atrial fibrillation which was present in five of the seven patients with congestive failure in the present study. Data from several studies (3, 44, 45) have suggested that the occurrence of this arrhythmia is related to the age of the patient with thyrotoxicosis and hence with the possibility of antecedent or underlying heart disease. The absence of this arrhythmia in the patients without heart disease or congestive failure (Group IA) and its occurrence in four of the six patients with heart dis-

ease but without congestive failure (Group IB) are consonant with this view. The occurrence of atrial fibrillation in the two younger patients (S. S., G. F.) who recovered from congestive failure and from the arrhythmia without evidence of heart disease, however, suggests thyrotoxicosis as the immediate cause perhaps because of increased myocardial sensitivity to epinephrine (39). The persistence of the arrhythmia in these two patients for over two years after they had become euthyroid and its eventual subsequent disappearance in one (S. S.) suggests, however, a disorder of a more chronic nature. Althought atrial fibrillation does not compromise the circulation at rest, impaired circulatory responses to the increased circulatory demands of exercise are well-documented (46–47). Since the circulation of the hyperthyroid patient is under a constantly increased load even at rest, the probable importance of this arrhythmia as a factor in the development of congestive failure is evident (48–49).

In addition to the direct alteration of myocardial function in the presence of excess thyroxin, the probable operation of a considerably increased peripheral circulatory load in these patients is suggested by the occurrence of heart failure at absolute levels of cardiac output which were normal or high. The "normal" cardiac outputs of the patients with antecedent heart disease (Group IIB; M. He., N. S., H. K.) and of Patient S. S. (Group IIB) who apparently had no antecedent or residual heart disease were inadequate with respect to the increased oxygen requirement of thyrotoxicosis; the ratio of cardiac output to oxygen consumption was abnormally low and the arteriovenous oxygen difference abnormally widened. The cardiac outputs of the other three patients (Group IIB) at rest, however, were more than adequate with respect to this usual measure of circulatory load since the ratio of cardiac output to oxygen consumption was increased and the arteriovenous oxygen difference abnormally narrowed. Since clinical congestive failure had occurred and the hemodynamic responses observed in these individuals during exercise were compatible with myocardial inadequacy, an additional peripheral nonoxygen load may be postulated for which their cardiac outputs were inadequate. The existence of a nonoxygen circulatory load (10, 19, 50, 51) in uncomplicated thyrotoxicosis is also suggested by the elevation of the ratio of cardiac output to oxygen consumption and narrowed arteriovenous difference found in the patients without heart disease or congestive failure (Group IA).

The nature of a peripheral nonoxygen circulatory load remains speculative. The evidence is excellent that many of the cardiovascular phenomena associated with excess thyroxin may be due in considerable part to hyper-reactivity to catechol amines and peripheral sympathomimetic hypersensitivity (52). It is difficult, however, to attribute the warm, flushed skin and decrease in total systemic resistance found in many thyrotoxic patients solely to chronically increased sympathetic stimulation or reactivity.

The peripheral circulatory stigmata of thyrotoxicosis in man, in contrast to dogs, whose chief avenue of heat loss is through the lungs, have suggested that a considerable nonoxygen load in this disorder may be attributable to an abnormally increased skin and peripheral blood flow caused in part by increased heat loss requirements (53–57). The hemodynamic data in these thyrotoxic patients resemble in many important respects the data obtained in patients with systemic arteriovenous fistulae studied in our laboratory (58). In these patients, in whom the circulatory load is obviously restricted to the low resistance lesion in the peripheral circulation, an abnormally high ratio of cardiac output to oxygen consumption and narrowed arteriovenous difference were found both in patients with and those without congestive heart failure and the changes observed in cardiac output and intravascular pressures during exercise were similar to the corresponding groups in the present study.

Other compensatory consequences of systemic arteriovenous fistulae and of thyrotoxicosis are also similar. An increase in blood volume frequently occurs in thyrotoxicosis and is commonly found in patients with arteriovenous fistulae (58–61). In both of these conditions edema may occur and indeed simulate congestive failure (62, 63) in the presence of quite normal hemodynamic responses to exercise. Edema and a 35 per cent increase in blood volume were found despite an adequate cardiac output response to exercise and normal atrial pressure in Patient L. P. (Group IB) who had rheumatic heart disease and had

transient bouts of atrial fibrillation. The continued development of hypervolemia, which initially represents a desirable circulatory adjustment, may well be a factor in the development of myocardial inadequacy (64) because of an augmented venous return, overdistention of the heart, and perhaps by contributing to the development of atrial fibrillation. Since the finding of an inadequate cardiac output response to exercise in the patients in Group IIB clearly indicates that actual myocardial inadequacy may develop in patients with thyrotoxicosis, as in patients with systemic arteriovenous fistulae, at remarkably high absolute levels of cardiac output, it is evident that the term "noncardiac circulatory congestion simulating congestive failure" (62) may not characterize adequately the abnormalities of the circulation in patients with these disorders.

The data in this report suggest that congestive heart failure may occur in the course of thyrotoxicosis by several mechanisms. Inadequate myocardial function with respect to the increased circulatory load may result from a direct effect of thyroxin on the heart in the presence of sympathomimetic amines, the increased circulatory load of the disease may overcome a myocardium damaged by heart disease of other etiology and the increased circulatory load of thyrotoxicosis may of itself overcome the myocardial reserve of the apparently normal heart. When a decrease in myocardial reserve is the major factor the cardiac output tends to be in the "normal" range. When the decrease in myocardial reserve is less and the increase in circulatory load greater the cardiac output may be remarkably high.

Summary

1. Clinical and hemodynamic studies are reported in 20 patients with thyrotoxicosis, in seven of whom congestive failure had occurred.

2. In 14 patients without congestive failure the hemodynamic responses during exercise were qualitatively normal, despite the fact that six had antecedent heart disease.

3. In the patients who had been in congestive failure the absolute level of cardiac output was normal in four and was markedly elevated in three. All had inadequate responses of the cardiac output to exercise.

4. Atrial fibrillation occurred only in patients in congestive failure or in those with antecedent heart disease.

5. The peripheral circulatory load in thyrotoxicosis is hemodynamically similar to that of systemic arteriovenous fistulae.

6. Congestive failure occurred in the presence of the circulatory load of thyrotoxicosis when myocardial function was reduced by heart disease of other etiology, when thyrotoxicosis transiently caused an inadequate myocardial function, or when the circulatory load apparently overcame the reserve of even the otherwise normal heart.

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EVALUATION OF NICOTINIC ACID AS AN HYPOCHOLESTEREMIC AND ANTI-ATHEROGENIC SUBSTANCE *

By MEYER FRIEDMAN and SANFORD O. BYERS with the technical assistance of WARREN HAYASHI, CLARENCE OMOTO and BETTY TSAI

(From the Harold Brunn Institute, Mount Zion Hospital and Medical Center, San Francisco, Cal.)

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Despite the gaps existing in our contemporary understanding of the origin, the function, the mode of regulation and even the eventual fate of plasma cholesterol, various drugs have been introduced and applied to lower this particular lipid in the blood stream of man. It is perhaps suggestive of the clinical eagerness to accomplish this lowering that the precise *modus operandi* by which any of these substances effect their reduction of cholesterol in blood still remains to be determined.

One of these substances is nicotinic acid. Its administration and subsequent cholesterol-lowering effect in clinical subjects was first reported by Altschul, Hoffer and Stephen (1), thereafter to be confirmed by Parsons and co-workers (2), and by Miller, Hamilton and Goldsmith (3). Administration of this substance to rats (4) and rabbits (5) also has been followed by a reduction in serum cholesterol. Thus there appears to be little doubt that the oral administration of nicotinic acid is capable of reducing the serum cholesterol of both the normo- and the hypercholesteremic subject and laboratory animal when no dietary control is exercised.

Interested in the possible mechanism of this action, we performed various studies upon both the rat and the rabbit. The results, as we shall indicate below, suggest that the chief efficacy of the drug in regard to the cholesterol dynamics of the rat and rabbit appears to reside in its anoretic properties.

METHODS

A. Effect of nicotinic acid upon intestinal absorption of cholesterol and lipid by the rat. Ten rats (Long Evans strain) were maintained for 12 days on regular Purina Lab Chow® to which nicotinic acid (1 per cent) had been added. At the end of this period, each rat was given 150 mg. of cholesterol in 3 ml. of olive oil, anesthetized, and the intestinal lymph duct was cannulated (6). Lymph was collected for 24 hours and then analyzed for total cholesterol (7) and total lipid (8). Ten control rats given only regular Purina Lab Chow® were subjected to the same procedures. Plasma samples obtained on the first and twelfth day were analyzed for total cholesterol (9).

B. Effect of nicotinic acid on biliary excretion of cholesterol and cholate in the rat. Ten rats fed regular Purina Lab Chow® containing nicotinic acid (1 per cent) for 14 days were subjected to cannulation of their bile duct and the 24 hour volume of bile was analyzed for total cholesterol (10). An analogous bile collection was done upon 10 control animals given Purina Lab Chow® alone.

For the determination of biliary excretion of bile cholate, five rats were fed Purina Lab Chow® containing nicotinic acid (0.5 per cent) and in addition given 50 mg. of nicotinic acid by stomach tube daily for three days. The bile duct was then cannulated and the 24 hour bile was collected and analyzed for cholate (11). Control bile collections were done with four animals fed only Purina Lab Chow®.

C. Effect of nicotinic acid upon plasma cholesterol of rats fed a high fat-cholesterol diet. Four groups of 10 rats each were studied. The first group of rats (controls) was allowed to ingest *ad libitum* a high fat-cholesterol diet [1] which was slightly modified from that described by Hartroft (12). The second group of rats was allowed to ingest *ad libitum* the same diet containing in addition nicotinic acid (1 per cent). The third and fourth groups, however, were fed differently because the earlier experiment had indicated to us that rats fed a diet containing nicotinic acid rarely ingest more than 5 to 10 Gm. of food per rat per day. To be certain, therefore, that the control rats were not ingesting more food than the rats given nicotinic acid, both groups were allowed to eat only 5 to 6 Gm. of food per rat per day. The food, however, ingested by the fourth group contained in addition nicotinic acid (1 per cent). It was observed that the daily ration invariably was consumed completely by both groups.

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[1] Butter, 40 per cent; casein, 20 per cent; cholesterol, 5 per cent; cellulose, 4 per cent; salt mixture, 4 per cent; vitamin mixture, 2 per cent; choline chloride, 1 per cent; thiouracil, 0.2 per cent; cholic acid, 0.3 per cent; sucrose: sufficient to make 100 per cent.

TABLE I

Effect of nicotinic acid on cholesterol and total lipid absorption

No. of rats	Average wt.		Plasma cholesterol		Lymph				
	Beg.	12 Days	Beg.	12 Days	Vol.	Total cholesterol		Total lipid	
	Gm.		*mg./100 ml.*			*mg./100 ml.*	*mg./24 hrs.*	*mg./100 ml.*	*mg./24 hrs.*
A. Rats given nicotinic acid									
10	293	290	52	39	44.0	59	24.5	3,522	1,444
S.E. of mean			±3.2	±1.9	±5.1	±5.2	±2.1	±103	±99
B. Control rats									
10	293	318	53	49	33.0	75	25.1	4,084	1,367
S.E. of mean			±2.54	±2.76	±1.7	±3.3	±1.9	±110	±84

The feeding program was continued for four weeks with weighings and plasma cholesterol determinations obtained weekly.

D. The effect of nicotinic acid upon plasma cholesterol and aortic atherosclerosis of rabbits fed a high fat-cholesterol diet. Twenty male rabbits, California Standard strain (approximately 10 weeks old), were given Purina Rabbit Chow® enriched with cottonseed oil (2 per cent) and cholesterol (1 per cent). Ten of these rabbits also received nicotinic acid (0.5 per cent) in their diet. Each series of animals was pair-fed as described previously (13) to ensure equality of ingestion. This program of feeding was continued for three months with weighings and plasma cholesterol determinations obtained monthly. At the end of three months, all animals were sacrificed. The aorta was assessed grossly for degree of atherosclerosis (13) and then an 8 cm. segment of aorta immediately distal to the attachment of the semilunar valves was analyzed for its cholesterol content (13).

RESULTS

A. Effect of nicotinic acid upon intestinal absorption of cholesterol and lipid of the rat

Rats given nicotinic acid in their regular chow for 12 days exhibited a moderate decline in their average serum cholesterol as compared to that of the controls (see Table I). Such rats, however, also failed to gain weight, an observation which led to the later dietary studies described below.

Regardless of the decline in serum cholesterol observed, the rats maintained on the nicotinic acid containing diet were found (see Table I) to absorb exogenously derived cholesterol and total lipid as well as the control animals.

B. Effect of nicotinic acid upon biliary excretion of cholesterol and cholate in the rat

Here again, the rats given the nicotinic acid containing diet for 14 days exhibited a slightly lower serum cholesterol and a lesser gain in weight than the controls (see Table II). However, the daily biliary excretion of cholesterol was almost identical in the experimental and control series.

Similarly, the biliary excretion of cholate in the rats given nicotinic acid for 72 hours prior to cannulation of the bile duct was approximately the same as that observed in the controls (see Table III). It is of course possible that the changes effected in cholate metabolism and excretion by the method of collection itself might have been of an order to obscure the possibly slight changes pro-

TABLE II

Effect of nicotinic acid on bile cholesterol

No. of rats	Average wt.		Plasma cholesterol		Bile		
	Beg.	14 Days	Beg.	14 Days	Volume	Cholesterol	
	Gm.		*mg./100 ml.*		*ml./24 hrs.*	*mg./100 ml.*	*mg./24 hrs.*
A. Rats given nicotinic acid							
10	295	310	56	47	19.5	15.7	3.0
S.E. of mean			±2.2	±3.5	±1.1	±1.6	±0.37
B. Control rats							
10	298	330	49	55	16.6	19.5	3.1
S.E. of mean			±3.0	±2.9	±1.7	±1.3	±0.21

TABLE III

Effect of nicotinic acid on bile cholate

No. of rats	Average weight	Bile		
		Vol.	Cholate	
	Gm.	*ml.*	*mg./100 ml.*	*mg./24 hrs.*
A. Rats on nicotinic acid				
5	290	18.4	212	39.0
S.E. of mean				(±1.3)
B. Control rats				
5	288	17.5	241	42.1
S.E. of mean				(±3.2)

duced by nicotinic acid administration. We believe, however, that this is most unlikely in view of the rather profound changes observed in cholate excretion in rats fed excess unsaturated oils (14) when studied by the same technique.

C. Effect of nicotinic acid upon plasma cholesterol of rats fed a high fat-cholesterol diet

Rather surprising results were observed in the feeding studies. Rats ingesting, *ad libitum*, the high lipid diet containing nicotinic acid exhibited throughout the period of feeding (see Table IV) a significantly reduced plasma cholesterol as compared with that observed in the control rats eating the same diet without nicotinic acid. However, these experimental rats were observed not only to eat less than the control rats but also to lose weight more sharply, and to fail to regain their original weight during the four weeks.

On the other hand, the series of rats that were pair-fed, and thus ingesting a limited quantity of this same diet plus nicotinic acid equal to that ingested by a control series of the same diet without nicotinic acid, after the first week exhibited (see Table IV) approximately the same weight and cholesterol changes as observed in the controls. Even the difference observed the first week is of doubtful statistical significance. Therefore, when intake of food was equalized, the addition of nicotinic acid did not exhibit a chronic hypocholesteremic effect.

D. Effect of nicotinic acid upon plasma cholesterol and aortic atherosclerosis of rabbits fed a high fat-cholesterol diet

Although it is not unusual for a third of a group of rabbits fed a high cholesterol-oil diet to succumb

TABLE IV

The effect of nicotinic acid on plasma cholesterol of rats given limited and unlimited intakes of high fat-cholesterol diet

Type of feeding	No. of rats	Av. daily food consumption throughout exp.	Onset		1 Week		2 Weeks		3 Weeks		4 Weeks	
			Wt.	Plasma cholest.	Wt.	Plasma cholest.	Wt.	Plasma cholest.	Wt.	Plasma cholest.	Wt.	Plasma cholest.
		Gm.	*Gm.*	*mg./100 ml.*	*Gm.*	*mg./100 ml.*	*Gm.*	*mg./100 ml.*	*Gm.*	*mg./100 ml.*	*Gm.*	*mg./100 ml.*
1. Unlimited intake + 1% nicotinic acid	10	8.3	304	62	261	144	267	330	277	346	274	284
S.E. of mean				±4.1		±8.4		±10.4		±12.3		±24
2. Unlimited intake	10	12.8	295	63	280	190	302	515	304	559	302	480
S.E. of mean				±3.8		±12.2		±12.1		±14.2		±33
3. Limited intake + 1% nicotinic acid	10	5.8	287	56	246	110	218	194	240	242	238	358
S.E. of mean				±3.2		±8.6		±11.1		±8.2		±21
4. Limited intake	10	5.8	286	59	255	148	225	162	220	242	227	317
S.E. of mean				±4.2		±9.4		±8.3		±9.2		±40.0

TABLE V

The effect of nicotinic acid on plasma and aortic cholesterol of pair-fed rabbits given high fat-cholesterol

No. of rabbits		Weight and plasma cholesterol						Aorta	
		Onset	1 Month		2 Months		3 Months		
Beginning	End	Wt.	Plasma cholest.	Wt.	Plasma cholest.	Wt.	Plasma cholest.	Grade atherosel.	Cholesterol
		Gm.	*mg./100 ml.*	*Gm.*	*mg./100 ml.*	*Gm.*	*mg./100 ml.*	*Gm.*	*mg./100 Gm.*	(0–4)	*mg./100 Gm.*
A. Rabbits given 1% cholesterol—2% cottonseed oil + nicotinic acid (0.5%)									
10	5	1,712	71	2,089	387*	2,489	810*	2,510	749*	0.9	1,812*
S.E. of mean			±6.1		±39.6		±96		±51		±184
B. Rabbits given 1% cholesterol—2% cottonseed oil only									
10	7	1,668	76	2,106	429	2,244	1,035†	2,436	1,020†	0.8†	1,950†
S.E. of mean			±4.9		±69.1		±66		±157		±220

* Five surviving animals.
† Seven surviving animals.

to intercurrent infections during a period of three months, half of the rabbits fed the nicotinic enriched experimental diet died of intercurrent infections manifested chiefly by nasal discharge, middle ear disorder and weight loss. Only three of the 10 control rabbits similarly succumbed. Certainly it was our impression that the rabbits fed nicotinic acid, as a group, presented a far less healthy and vigorous appearance than the controls.

The series of rabbits ingesting the high lipid diet with nicotinic acid had a monthly average plasma cholesterol which was consistently lower than that of the control rabbits. However, this difference could not be adjudged significant when subjected to statistical analysis. Thus for the three successive months (see Table V) the standard error of the difference of means was only 0.52, 1.92 and 1.7 times the difference of means, respectively. Similarly, the gross degree of aortic atherosclerosis and the average cholesterol content of the aortic samples were approximately the same in both series.

DISCUSSION

The results of the above studies are remarkably similar to those we recently obtained (13) in our study of the mode of action and possible effectiveness of potassium iodide as a hypocholesteremic and anti-atherogenic agent. Both substances were found ineffective in influencing the intestinal absorption of either cholesterol or total lipid when both experimental and control animals were given *precisely* the same amount of cholesterol and olive oil by stomach tube. Apparently, then, the hypocholesteremic effect of nicotinic acid does not stem from any interference with the absorption of either cholesterol or total lipid.

Again, similar to potassium iodide, the administration of nicotinic acid to the rat was not observed to influence the hepatic rate of cholesterol turnover as determined by the biliary cholesterol assay method (15). We employed this technique, rather than any radioactive tracer method, because at the time this work was done such methods were still yielding equivocal results when used as an indicator of the rate of synthesis of cholesterol (16, 17). The conflicting findings of Duncan and Best (4), compared with those of Merrill (18) (both employed radioactive tracers), concerning the rate of hepatic synthesis of cholesterol after adminis-

tration of nicotinic acid, serve to stress this difficulty in employing tracers for this particular determination. Nicotinic acid also was not found to effect the biliary excretion of cholate in our rats.

Unlike the administration of potassium iodide, that of nicotinic acid was found to lower the plasma cholesterol slightly but significantly in normocholesteremic rats when ingesting ordinary laboratory rat chow *ad libitum*. Such rats, however, lost weight, a phenomenon we concluded to be due to their decreased intake of food. The importance of this latter phenomenon in effecting the comparative hypocholesteremia observed was made clear in our subsequent feeding experiments with high fat-high cholesterol diets. In these experiments, when the control rats were allowed to ingest no more food than the experimental rats, no sustained hypocholesteremic effect resulted from the administration of nicotinic acid. However, the comparative hypocholesteremic effect of nicotinic acid promptly reappeared in the series of experimental and control rats that were allowed to ingest *ad libitum* the same type of diet. But here again, the rats on the nicotinic acid regimen ate less and lost weight. The likely conclusion thus appeared to be that nicotinic acid was acting as a hypocholesteremic agent because of its anoretic properties.

Finally, the results of nicotinic acid administration in the rabbit appeared similar to those obtained after the administration of potassium iodide. When the intakes of both the experimental and control series were made the same, the hypocholesteremic effect of nicotinic acid did not appear to be statistically significant. Also, no prevention of atherosclerosis was observed in the experimental animals.

The preceding results are opposite to those found both by Altschul and associates (1) and Merrill and Lemley-Stone (5). Neither of these authors, however, pair-fed their animals, although Altschul administered equal quantities of cholesterol to both experimental and control animals. We believe that in their studies, the anoretic properties of nicotinic acid reduced the quantity of either cholesterol or fat taken in by their experimental animals, hence effected the plasma, hepatic and aortic cholesterol values observed. Certainly the comparatively small amount of excess cholesterol present in the liver of nicotinic-acid-fed-rabbits of Merrill and Lemley-Stone (5) suggests that these animals were absorbing less exogenous cholesterol because this organ, at least as observed in the rat (19, 20), serves as the initial site of deposition of intestinally absorbed excess cholesterol. It also should be stressed, as we earlier observed (13), that the weights of rabbits cannot be employed as an exact indicator of their food intake.

If, then, nicotinic acid appears to act as an anoretic agent in its relationship to hypocholesterolemia and subsequent prevention of atherosclerosis in the experimental animal, can it exert a more specific effect in human lipid metabolism? In this connection, although Altschul and co-workers (1) reported a fall in the serum cholesterol of man a few hours after administration of a single dose of nicotinic acid, this could not be confirmed by Parsons and associates (2). Moreover, the clinical studies to date of which we are aware (1–3, 21, 22) have not subjected the patient to a strictly controlled regimen in which the actual quantity of the food ingested before, during and after nicotinic acid administration has been accurately determined. In addition, the weight changes of these patients have not been published, although Galbraith, Perry and Beamish (22) state that, without giving any actual data, their series of subjects showed no weight changes. Finally, in view of the frequency with which nicotinic acid induces nausea and other gastrointestinal effects (2, 21), it is of paramount interest to determine any possible spontaneous *qualitative* changes in food intake induced by its administration. It is a well-known clinical truism that a potential nauseant operating possibly still at a subclinical level might induce a reversion to foods of a lighter, less lipid containing nature. If this possible factor were in action, then the moderate hypocholesteremia ensuing after the administration of nicotinic acid becomes readily understandable. Certainly the relatively tremendous quantity of nicotinic acid needed for effective lowering of plasma cholesterol militates against the view that it functions in its usually accepted vitamin function. Even more disturbing is the observation that its use appeared to be of doubtful or slight value in a severe hypercholesteremia stemming from an undoubted endogenous defect such as idiopathic familial hypercholesteremia with xanthoma tuberosum (2).

When substances which are potential, as well as actual, disturbants of the gastrointestinal sys-

tem are under investigation, scrutiny of the sequences of events occurring in this system is justified fully as much as more detailed investigations of intracellular mechanisms residing in the tissues of more distant organs.

SUMMARY

Ingestion of nicotinic acid was not found to alter the rate of intestinal absorption of cholesterol or total lipid in the rat. No change either was observed in the rate of hepatic synthesis of cholesterol (as measured by the biliary cholesterol assay method) or in the biliary secretion of bile acid in the rat administered nicotinic acid. Finally, when the intake of a high fat-cholesterol diet was controlled in both the treated and control animal, the ingestion of nicotinic acid did not hinder the expected onset of hypercholesteremia.

Rabbits pair-fed a high cholesterol-fat diet with and without nicotinic acid failed to exhibit a significant difference either in their average plasma cholesterol or in their degree of aortic atherosclerosis.

It is suggested that the previously observed hypocholesteremic and atherosclerosis preventing properties of nicotinic acid may be due to an anoretic effect. The possible influence of this substance both upon the quantity and the *quality* of food taken by human subjects eating under noncontrolled conditions is discussed.

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THE RELATION OF PLASMA AND BILIARY CHOLESTEROL TO BILE ACID SYNTHESIS IN MAN *

By R. S. ROSENFELD and LEON HELLMAN

(From the Sloan-Kettering Institute for Cancer Research, New York, N. Y.)

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Bile is an important excretory route of steroids since it contains cholesterol and bile acids which are derived from cholesterol. That cholic acid is formed from cholesterol was demonstrated by Bloch, Berg and Rittenberg over a decade ago (1). Other workers have confirmed this finding in animals (2–6) and the conversion has also been demonstrated in the human (7, 8). Intermediates involved in this transformation have been extensively studied and reviewed by Bergström and Borgström (9, 10) and these workers have more recently reported on the stereochemical course of the transformation of cholesterol to cholic acid (11). This investigation deals with the dynamic aspects of the conversion of cholesterol of both endogenous and exogenous origins to cholic acid in man.

Cholesterol-4-C^{14} and sodium acetate-H^3 were fed to one subject, and sodium acetate-2-C^{14} was administered to a second subject; both had complete bile fistulas. It was found that orally ingested cholesterol and biosynthetic cholesterol mixed indistinguishably in the plasma after a short equilibration period. Plasma and biliary cholesterol had the same specific acivity and were in isotope equilibrium with the pool which served as the precursor of cholic acid.

EXPERIMENTAL

Clinical material. The subjects were hospitalized on a metabolic ward where complete collections of urine, feces and bile could be made. Subject A was a 58 year old white man who had an external bile fistula established surgically for the relief of a bile duct obstruction due to carcinoma of the pancreas. The completeness of the fistula was demonstrated by retrograde cholecystiography and acholic stools. At the time of the study he was in good clinical condition; the daily volume of bile drained through the fistula varied from 630 to 850 ml. This patient received approximately 2 Gm. of bile salts daily. Subject B was a 60 year old white man in whom the bile fistula was established during an operative attempt to resect a carcinoma of the pancreas. The completeness of the fistula was demonstrated by acholic stools. At the time of the study this patient was in fair clinical condition; he was slightly jaundiced and the serum bilirubin was 1.60 mg. per cent; other liver function tests were normal with a cephalin flocculation of 1 + and a thymol turbidity of 0.6. The output of bile varied from 520 to 650 ml. daily. Subject A received 15.4 mg. of cholesterol-4-C^{14} (5.9 μc. per mg.) and 700 μc. of sodium acetate-H^3. Subject B received 309 μc. of sodium acetate-2-C^{14}. All labeled material was given by mouth after an overnight fast.

Isolation procedures. Blood and bile were collected at predetermined time intervals from each patient; feces obtained in a 24 hour period were combined. Free and ester cholesterol were isolated from plasma by a method described previously (12). Bile samples were acidified with sulfuric acid to pH 1 and continuously extracted with ether for 48 hours; the ether solution was extracted with 5 per cent sodium hydroxide. The ether solution was then concentrated and the residue was chromatographed on alumina. Cholesterol was eluted by petroleum ether-benzene mixtures and after recrystallization from acetone, melted at 148 to 149°. The aqueous alkaline solution was treated with carbon dioxide until a copious precipitate of sodium carbonate appeared in the flask. Water was added in sufficient quantity to dissolve the precipitate and the solution was extracted with ether to remove phenolic material. The residual alkaline solution was acidified with hydrocholoric acid and, after evolution of gas, extracted with ethyl acetate. The ethyl acetate solution was concentrated to obtain the crude conjugated bile acid fraction. This material was dissolved in 10 per cent potassium hydroxide and refluxed for 16 hours. After cooling, the alkaline solution was washed with ether. Acidification of the alkaline layer followed by ethyl acetate extraction yielded a solution of crude bile acids which partially crystallized on removal of the solvent. This material was dissolved in 1 : 1 methanol-ethyl acetate and was esterified with diazomethane in ether solution. Concentration of this solution gave an oil which was chromatographed on silica gel (13). Two principal fractions were obtained from the column. Material eluted with ether was shown by infrared spectrometry to be a

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mixture of methyl deoxycholate and methyl chenodeoxycholate. Crystalline methyl cholate was obtained from the acetone-ether (1:1) eluates. This material had an infrared spectrum identical with that of authentic methyl cholate. Recrystallization from ethanol-petroleum ether yielded methyl cholate, melting point 156 to 157°; the melting point did not change on admixture with authentic material.

Cholic acid content of whole bile was quantitatively determined by the method of Irvin, Johnston and Kopala (14).

Feces were homogenized with 500 ml. of acetone and centrifuged; the supernatant was decanted. This extraction procedure was repeated three times. The combined acetone supernatants were concentrated and then saponified by refluxing for three hours in 20 per cent alcoholic potassium hydroxide. The nonsaponifiable material was extracted with petroleum ether and the oil which was obtained was chromatographed on alumina for the isolation of cholesterol and coprostanol.

The radioactivity of cholesterol was measured by depositing cholesterol digitonide on stainless steel planchets from an acetone-alcohol slurry (12). Methyl cholate and coprostanol were melted onto the planchets. Carbon-14 activity of these samples containing both radiocarbon and tritium was determined in a windowless flow-gas counter with the tritium beta particles screened out by aluminum foil (15). Tritium activity was determined by converting the samples to hydrogen gas which was counted in a Geiger tube (16). The radiocarbon counting procedure was calibrated against a National Bureau of Standard C^{14} standard and the carbon radioactivity data are expressed in terms of distintegrations per minute per millimole (DPM/mMole). The tritium radioactivity data are given in terms of counts per minute per millimole of hydrogen (cpm/mMole H_2).

RESULTS AND DISCUSSION

Figures 1 and 2 demonstrate the biosynthesis of plasma and biliary cholesterol from sodium acetate in Subjects A and B, respectively. The maximum radioactivity of plasma free and biliary cholesterol was attained at three to four hours after administration of the dose. The specific activity of the early samples of cholesterol isolated from bile exceeded that of plasma cholesterol by about 10 per cent. The lower plasma values may be explained by the dilution of newly synthesized labeled cholesterol by the pre-existing pool of nonradioactive sterol in the plasma; the difference between the radioactivity of plasma and biliary cholesterol disappeared after two days. Bile cholesterol which drained directly to the exterior did not undergo this dilution and was perhaps more representative of newly synthesized liver cholesterol. Rosenman

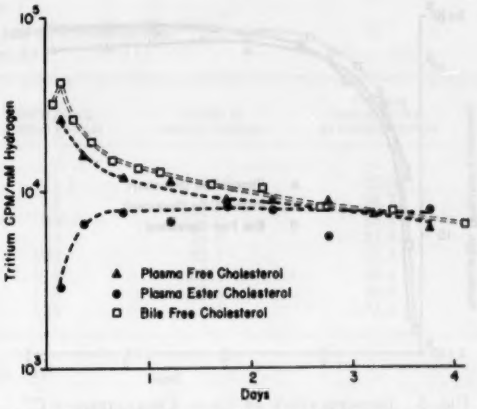

FIG. 1. INCORPORATION OF ACETATE-2-H^3 INTO PLASMA AND BILIARY CHOLESTEROL

and associates (17, 18) have also pointed out that the cholesterol which appears in the bile of their experimental animals represents newly synthesized sterol. A similar elevation in the specific activity of biliary cholesterol as compared with plasma cholesterol was reported by Gould and coworkers who administered labeled acetate to dogs with bile fistulas (19).

The simultaneous appearance of radioactivity in bile and plasma cholesterol and the similarity of the specific activity curves indicate that biliary and plasma cholesterol are in rapid isotope equilibrium with a common pool. As would be expected, liver cholesterol also forms part of this common pool since it has been found from human

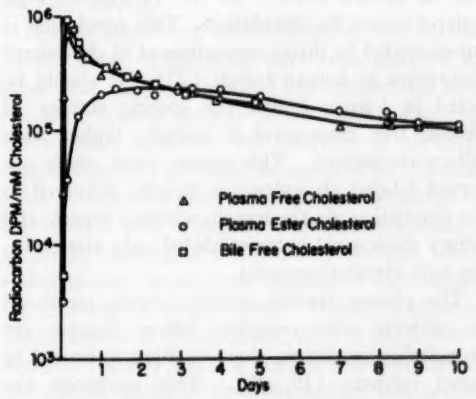

FIG. 2. INCORPORATION OF ACETATE-2-C^{14} INTO PLASMA AND BILIARY CHOLESTEROL

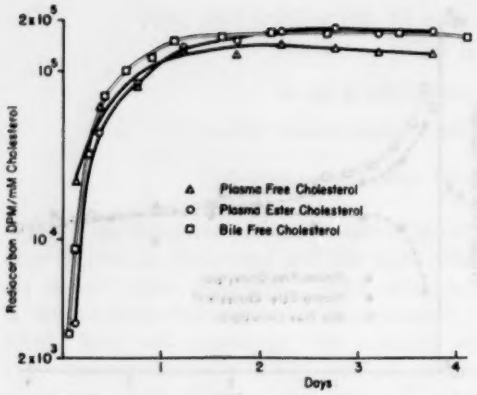

FIG. 3. INCORPORATION OF ORAL CHOLESTEROL-4-C^{14} INTO PLASMA AND BILIARY CHOLESTEROL

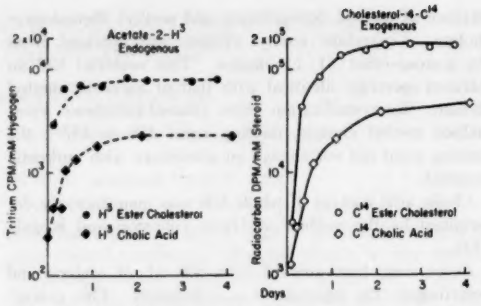

FIG. 4. SIMULTANEOUS INCORPORATION OF ACETATE AND CHOLESTEROL INTO BILE ACID AND PLASMA ESTER CHOLESTEROL

biopsy material that hepatic and plasma cholesterol have the same specific activity after administration of radioactive sodium acetate (20).

The cholesterol ester of plasma has an initially low radioactivity (Figures 1 and 2) which gradually increases so that its specific activity curve intersects those of the plasma and biliary free cholesterol at about two and one-half days (21).

The interrelations of plasma and biliary cholesterol derived from orally ingested cholesterol-4-C^{14} in Subject A are shown in Figure 3. The specific activities of plasma free, ester and biliary cholesterol are initially at low levels and reach a maximum at one to two days (15). This suggests that the absorption of cholesterol from the intestine is relatively prolonged with a gradual rise in specific activity as the radioactive cholesterol enters the circulation. This conclusion is substantiated by direct measurement of cholesterol absorption in human lymph (22). It should be noted in Figure 3 that the specific activity of plasma free cholesterol is initially higher than biliary cholesterol. This occurs since newly absorbed labeled cholesterol is directly delivered to the circulation via the lymph, whereas hepatic and biliary cholesterol become labeled only after mixing with circulating sterol.

The plasma specific activity curves measured in subjects with complete biliary fistulas are remarkably similar in slope to those measured in intact subjects (15, 21). This similarity extends as far as 10 days (Figure 2) after administration of the dose and suggests that the biliary loss of cholesterol occurring under the conditions of this experiment does not result in either increased biosynthesis or absorption of cholesterol in response to the potential diminution of the size of the common cholesterol pool. Had such changes occurred, a more rapid rate of fall-off in specific activity would have been expected due to the increased influx of unlabeled cholesterol.

Figure 4 compares the radioactivity of plasma ester cholesterol with that of cholic acid in Subject A. The parallel increase of tritium radioactivity in cholic acid and ester cholesterol suggests that both these substances are derived from the common sterol pool represented by the plasma, biliary and hepatic free cholesterol. The similarity in shape of the C^{14}-radioactivity curves derived from dietary cholesterol further suggests the precursor role of plasma free cholesterol. The specific activities of plasma ester cholesterol are considerably higher than corresponding values for cholic acid throughout the period of observation. This is to be expected since the specific activity of cholic acid was undoubtedly lowered by dilu-

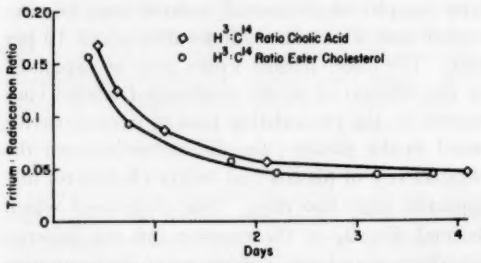

FIG. 5. TRITIUM : RADIOCARBON RATIOS IN ESTER CHOLESTEROL AND CHOLIC ACID

TABLE I

C^{14}-radioactivity in bile and in methyl cholate
(Subject A)

Duration of collection	Volume of bile	DPM*	Cholate in bile	DPM/mg.‡ methyl cholate	DPM* of methyl cholate	Per cent radioactivity as methyl cholate
hrs.	*ml.*		*mg.†*			
2	60	16.3	311	11.5	3.60	22.0
4	86	44.2	432	15	6.50	14.7
6	133	97.4	460	60	27.6	28.4
6	257	96.5	290	82	24.0	23.9
6	160	106	557	100	55.7	52.2
6	254	112	576	110	63.5	56.6
6	163	98	166	118	19.6	20.0
6	98	24.7	66.5	125	8.3	33.5

* Disintegrations per minute $\times 10^{-3}$.
† Measured by the method of Irvin, Johnston and Kopala (14).
‡ Specific activity of isolated material.

tion with the bile salts administered as replacement therapy; however, it is not possible to state unequivocally that this is the only reason for the difference. Despite this difference, the precursor-product relationship of the pool represented by plasma cholesterol and cholic acid can be seen unequivocally in Figure 5 where the isotope ratio of tritium to C^{14} is identical in plasma ester cholesterol and cholic acid. These data indicate that cholic acid is derived from cholesterol of both biosynthetic and exogenous origin.

In Table I, 15 to 56 per cent of the radioactivity in bile was present as cholic acid as measured by the method of Irvin and co-workers (14). These figures are lower than the values for biliary cholic acid reported by Siperstein and Murray (8). However, their subject was in good health except for the bile fistula and the cholesterol tracer dose was administered intravenously. These factors might account for the differences.

The radioactivity of cholesterol and coprostanol isolated from the feces of Subject A during the first day was compared with that of plasma free cholesterol at 24 hours (Table II). The carbon-14 specific activities of the fecal sterols are 200 times higher than plasma cholesterol-C^{14} which would be expected if a portion of orally ingested, highly radioactive sterol escaped absorption. The correspondingly high C^{14} activity in coprostanol shows that it was formed from cholesterol in the intestine. This observation is supported by experiments which demonstrate the conversion of cholesterol to coprostanol both in the intestine and in incubation studies with feces (23). However, the tritium specific activity of fecal cholesterol is quite similar to that of plasma free cholesterol. Because of the presence of the bile fistula, cholesterol could only enter the intestine by way of the mucosa. The data on this patient as well as other observations (24) suggest that plasma cholesterol secreted through the intestinal mucosa serves as an important precursor for fecal sterols.

TABLE II

Radioactivity of fecal sterols after one day
(Subject A)

	Coprostanol	(Feces) cholesterol	(Plasma free) cholesterol
T*		12.8	11.0
C^{14}†	7.7	10.0	0.03

* Disintegrations per minute (DPM) per millimole of hydrogen $\times 10^{-3}$.
† DPM per millimole $\times 10^{-7}$.

Several important relationships of the various steroid pools of the body have been clarified by these data. Plasma, bile and liver cholesterol have the same specific activity under equilibrium conditions and, therefore, the behavior of all three components will probably be involved in any situation in which there is a change in only one member of this pool. The enterohepatic circulation of biliary cholesterol is a pathway through which endogenously synthesized cholesterol can assume the character of dietary sterol. If some qualitative characteristic of dietary sterol, such as the physicochemical form in which it is absorbed, is related to the atherogenecity of cholesterol, then endogenous cholesterol should be considered in the same light as cholesterol of dietary origin.

SUMMARY

Sodium acetate-H^3 and cholesterol-4-C^{14} were administered orally to one subject and sodium acetate-2-C^{14} was given to a second subject; both had complete biliary fistulas. Cholesterol was isolated from the plasma and bile and cholic acid (as methyl cholate) was obtained from the bile. Sufficient samples were isolated to permit the construction of rate curves so that the dynamic behavior of these substances could be compared.

It has been found that biliary cholesterol and plasma free cholesterol are parts of the same metabolic pool. Evidence is also presented which shows that cholic acid is derived from cholesterol of endogenous and dietary origin. After ingestion of cholesterol-4-C^{14}, from 10 to 50 per cent of the C^{14}-radioactivity in bile was accounted for as cholic acid.

Cholesterol and coprostanol were also isolated from the feces and evidence has been presented to show that a significant portion of the fecal sterols are derived from plasma cholesterol and arise through intestinal excretion.

ACKNOWLEDGMENT

The support and interest of Dr. T. F. Gallagher in this investigation are gratefully acknowledged.

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THE EFFICIENCY OF VENTILATION DURING VOLUNTARY HYPERPNEA: STUDIES IN NORMAL SUBJECTS AND IN DYSPNEIC PATIENTS WITH EITHER CHRONIC PULMONARY EMPHYSEMA OR OBESITY *

By H. W. FRITTS, Jr., J. FILLER, A. P. FISHMAN and A. COURNAND

(From the Department of Medicine, Columbia University, College of Physicians and Surgeons, and the Cardio-Pulmonary Laboratory of the First Medical and Chest Services, Columbia University Division, Bellevue Hospital, New York, N. Y.)

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The ventilatory apparatus is analogous to a mechanical system composed of an engine coupled to a pump. The engine comprises the chest wall and the diaphragm, while the pump embraces the airways and the lungs. Although the two units operate as a single assembly, their functions are distinct. The engine converts metabolic energy into mechanical work; the pump translates this work into a rhythmic exchange of air.

According to this schema, the useful work of the chest wall and diaphragm is that performed on the surfaces of the lungs. Hence, the efficiency with which this work is accomplished is defined by the formula written below.

$$\text{Per cent efficiency} = \frac{\text{mechanical work performed on the lungs}}{\text{total energy used for breathing}} \times 100.$$

Using this formula, we have calculated the efficiencies of seven normal subjects, of six patients with chronic pulmonary emphysema, and of five patients who were obese. A presentation of these data is the purpose of this report.

METHODS

a) Estimation of the energy used for breathing. The energy used for breathing was estimated by using a modification (1) of the method of Liljestrand (2). In brief, the procedure entailed measuring the oxygen uptake first at rest, then at from 10 to 20 different levels of voluntary hyperpnea. At each level the subject breathed a gas mixture containing a small concentration of carbon dioxide; this prevented the development of hypocapnia and enabled the subject to maintain an augmented volume of ventilation for 20 minutes, a sufficiently long period for the establishment of a steady state. During the final two minutes of each period the ventilation was measured and collections of expired gas were made. Two samples of this gas were analyzed for oxygen and carbon dioxide; duplicate analyses were required to check within 0.03 volume per cent.

In each of the experimental periods, the subject breathed at a frequency of 30 breaths per minute by following the rhythm set by a metronome. This frequency was chosen because the subject could achieve a large minute volume of ventilation with a small tidal volume of air. Although the size of the tidal volume did not affect the measurement of energy, it appeared to be an important factor in the measurement of work. The reasons for this importance are outlined in Part *b* of this section; they indicated that measurements of work were most reliable when the tidal volumes were small.

The data from those experiments in which the respiratory quotients lay outside the range of 0.70 to 0.95 were discarded. The data from the remaining runs were plotted on coordinate axes, and the curve that provided the best visual fit was drawn through the points (Figure 1, A). Since such a curve did not directly indicate the total oxygen used for breathing, this quantity was estimated by following these steps: *1)* The oxygen used for quiet breathing at a frequency of 30 was calculated by multiplying the slope of the lowermost end of the curve by the resting ventilation. *2)* This product was subtracted from the resting oxygen uptake to define a baseline value. Then *3)* the total oxygen used for breathing at any level of ventilation was estimated by subtracting this value from the oxygen uptake measured during hyperpnea. In effect, this resolved the total oxygen uptake into two components: a nonrespiratory component which was assumed to stay constant, and a respiratory component which became larger as the ventilation increased.

The oxygen used for breathing was converted to equivalent kilogram-meters (Kg. M.) of energy by multiplying the respiratory component, expressed as cc. O_2 per minute, by the factor 2.06 (3). Implicit in the use of this factor were the premises that the oxygen was utilized solely for the combustion of fat and carbohydrate, and that these foodstuffs were consumed in such proportions that the respiratory quotient had an average value of 0.8.

b) Estimation of the mechanical work performed on the lungs. The mechanical work performed on the lungs

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was estimated from the areas of pressure-volume diagrams. For the construction of these diagrams it was necessary to know the change in the volume of the lungs and the difference in pressure between the mouth and the pleural space. Changes in volume were measured by graphic integration of the signal of a Mead-Silverman pneumotachygraph. A preliminary study of this instrument indicated that its response was linear for flow rates between 0 and 190 L. per minute, and that its calibration was not perceptibly altered when the fraction of oxygen in the calibrating mixture was varied between 16 and 25 per cent, and the fraction of carbon dioxide between 0 and 8 per cent. But despite this insensitivity to changes in gas composition, other factors—notably temperature, water vapor and the respiratory quotient—caused the inspired and expired volumes to differ by sufficient amounts to preclude the construction of closed-loop pressure volume diagrams. The effects of these factors, previously analyzed by Mead and Whittenberger (4), were such that when dry gas was inspired at a temperature of 24° C. and under a pressure of one atmosphere, the change in the volume of the lungs was about 10 per cent greater than the volume of air entering the mouth. During expiration the temperature of the gas leaving the mouth was about 32° C. (4), and the volume measured by the pneumotachygraph was about 3 per cent smaller than the actual change in the size of the lungs. Thus, the expired volume usually exceeded that inspired by approximately 7 per cent.

To minimize the disparity between these volumes, we converted each to a B.T.P.S. value by using the measured temperature of the inspired gas mixture and by using 32° C. as the approximate temperature of the expired gas. In most instances these conversions minimized the difference between the volumes to the extent that the small remaining discrepancy could be ignored.

The other variable needed to construct pressure-volume diagrams was the pressure-difference between the mouth and the pleural space. For this value we substituted the pressure-difference between the mouth and the lumen of the esophagus. To test the validity of this substitution, we followed the procedure of others (5–9) and compared the two pressure-differences in nine patients with pneumothorax. The intraesophageal pressure was recorded through a No. 7 water-filled cardiac catheter which, after being softened by heating in warm water, was advanced through the nose until the tip lay at the level of the pulmonary artery. The pressure in the esophagus referred to atmosphere was measured by connecting the catheter to a Statham gage, the level of the gage having previously been adjusted to the height of the angle of Louis. Because the catheter was filled with water, a correction had to be made for the difference in heights of the tip of the catheter and the tip of the gage. To make this correction, a lateral X-ray of the chest was taken with a plate of three-fourths inch lucite interposed between the body of the subject and the X-ray cassette. The lucite plate contained a grid of radiopaque brads, and the images of the brads were superimposed on the X-ray film of the lateral view of the chest. Since these brads were spaced at one centimeter distances, the difference in heights of the gage and the angle of Louis could be determined by drawing horizontal lines through the levels and counting the brad spaces between the two. This distance, measured in centimeters, was added to the gage pressure to give the pressure in the esophagus referred to atmosphere.

For the measurement of intrapleural pressure, a No. 14 trocar was inserted through the chest wall and a polyethylene tube of 2 mm. internal diameter was threaded through the lumen. After removing the trocar, we recorded the intrapleural pressure by attaching the polyethylene tube to a second Statham gage. The system used to record this pressure had characteristics similar

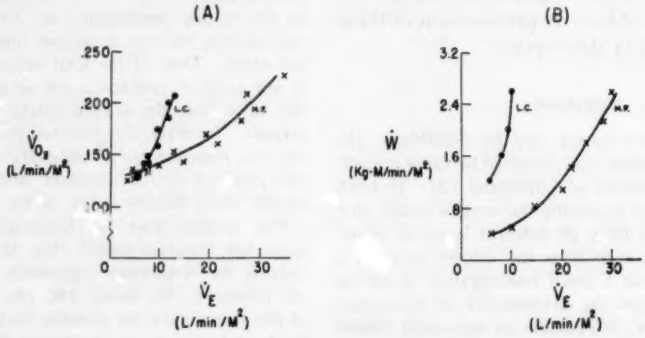

FIG. 1. RELATION BETWEEN OXYGEN UPTAKE ($\dot{V}_{O_2}$) AND VENTILATION ($\dot{V}_E$) IN H.F., A NORMAL SUBJECT, AND IN L.C., A PATIENT WITH CHRONIC PULMONARY EMPHYSEMA (A); AND RELATION BETWEEN MECHANICAL WORK PERFORMED ON THE LUNGS ($\dot{W}$) AND VENTILATION ($\dot{V}_E$) IN THE SAME TWO SUBJECTS (B)

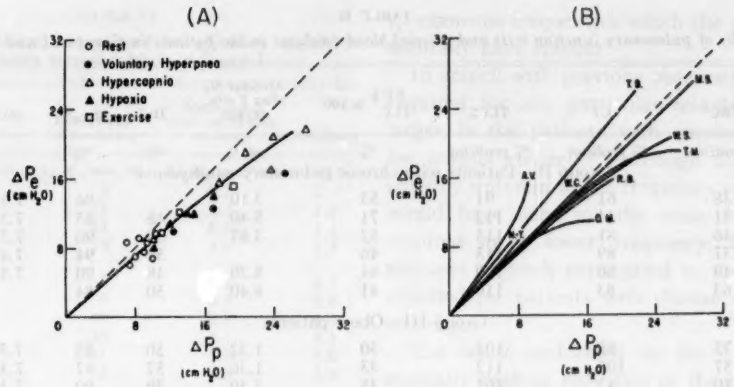

FIG. 2. COMPARISON OF SWINGS IN INTRAESOPHAGEAL PRESSURE (ΔP_e) WITH SWINGS IN INTRAPLEURAL PRESSURE (ΔP_P) IN SUBJECT W.S. DURING QUIET AND AUGMENTED VENTILATION (A) AND COMPARISON OF ΔP_e AND ΔP_P IN ALL NINE PATIENTS WITH PNEUMOTHORAX (B)

to those of the system used to measure the pressure in the esophagus. The natural frequency of the former was 39 cycles per second (cps), and of the latter, 28 cps.

All measurements were made with the subject supine. Diverse stimuli were employed to increase the ventilation, including exercise, the inhalation of 5 per cent CO_2, and the inhalation of 12 per cent O_2. In addition, each patient changed his minute volume of ventilation by voluntarily altering his rate and depth. With each stimulus or voluntary alteration, the pressures in the two spaces were compared in two ways: *1*) the magnitude of the swings in pressure (*i.e.*, the difference between peak inspiratory and peak expiratory pressures) averaged over at least 10 respiratory cycles, and *2*) the mean pressures obtained by planimetric integration of the pressure records.

Nine patients, each with either a spontaneous or an induced pneumothorax, were studied. The agreement of the swings in pressure are presented in Figure 2. The construction of the curve of agreement in Patient W.S. is shown in Figure 2, A, and the curves of all nine patients are shown in Figure 2, B. These curves indicated that the agreement was independent of the stimulus used to increase ventilation, and that the correlation was best at low tidal volumes when the swings in pressure were small. Because the correlation became less and less satisfactory as the tidal volume became larger, all studies of efficiency were carried out at a frequency of 30 breaths per minute. As previously mentioned, this frequency allowed the achievement of a large minute volume of ventilation with a small tidal volume of air.

In general, the correspondence between the mean pressures in the two spaces was poor. At rest the mean intraplueral pressure referred to the mouth varied between -1 and -5 cm. H_2O, while that in the esophagus varied between $+5$ and -3 cm. H_2O. Moreover, the relationship between the mean pressures in any single patient was frequently inconstant and was almost always altered, sometimes to a considerable degree, when the minute volume of ventilation was changed. For example, in the patient whose data are plotted in Figure 2, A, the mean pressure in the esophagus referred to the mean pressure in the pleura varied between -1 and $+5$ cm. H_2O when different stimuli were applied. These results support those published previously by Cherniack, Farhi, Armstrong and Proctor (9).

Because the correlation between the mean pressures was not satisfactory, pressure-volume diagrams were con-

TABLE I

Physical characteristics of the subjects in Groups I, II and III

Subject	Age	BSA	Wt.
	yrs.	*M.²*	*Kg.*
Group I—Normal subjects			
G.P.	32	1.91	75.1
H.F.	35	1.94	77.2
R.B.	30	2.10	91.3
J.F.	41	1.86	72.6
C.B.	19	1.62	53.6
A.S.	47	1.89	67.0
A.D.	56	2.04	84.8
Group II—Patients with chronic pulmonary emphysema			
T.B.	61	1.84	68.5
L.C.	64	1.53	45.4
W.L.	54	1.80	55.3
E.E.	66	1.78	64.6
D.G.	53	1.76	90.6
J.G.	49	1.77	70.6
Group III—Obese patients			
H.W.	36	2.13	137.0
G.W.	55	2.30	127.0
J.B.	40	2.34	120.0
M.S.	33	2.46	136.6
A.H.	61	2.48	141.0

TABLE II

Results of pulmonary function tests and arterial blood analyses in the patients in Groups II and III

Subject	MBC*	VC†	TLC‡	$\frac{RV}{TLC}$ § × 100	Alveolar N_2 after 7 min. oxygen	V_{Ht}	S_{aO_2}¶	pH_s**	P_{aCO_2}††
	% predicted	*% predicted*	*% predicted*	%	*vol. %*	%	%		*mm. Hg*
Group II—Patients with chronic pulmonary emphysema									
T.B.	38	61	91	53	3.10		96	7.41	35
L.C.	31	44	112	71	5.40	48	85	7.35	49
W.L.	46	81	115	52	3.87	39	90	7.33	46
E.E.	37	89	115	46		54	94	7.49	34
D.G.	49	80	100	44	8.20	48	90	7.51	30
J.G.	63	83	110	41	8.40	50	84		
Group III—Obese patients									
H.W.	75	88	102	30	1.32	50	85	7.39	45
G.W.	57	105	112	33	1.36	57	97	7.33	40
J.B.	70	82	107	38	2.39	39	90	7.39	47
M.S.	64	93	95	22	1.74	49	93	7.41	31
A.H.	50	75	112	52	3.79	46	89	7.44	37

* MBC = maximum breathing capacity.
† VC = vital capacity.
‡ TLC = total lung capacity.
§ RV = residual volume.
‖ Ht = hematocrit.
¶ S_{aO_2} = arterial blood oxyhemoglobin saturation.
** pH_s = serum pH.
†† P_{aCO_2} = arterial blood carbon dioxide tension.

structed in the conventional manner on the assumptions that: *1)* The pressure difference across the lungs at the start of inspiration was zero, and *2)* the muscles of the chest and abdominal walls contributed to the work of expiration only when the intraesophageal pressure during expiration exceeded that at the start of inspiration. The errors introduced by these assumptions are considered in the Discussion.

The values of work calculated for the different levels of hyperpnea were used to construct curves relating work to ventilation. Typical curves are shown in Figure 1, B.

c) The calculation of efficiency. Efficiency was calculated by dividing the mechanical work performed on the lungs per minute by the corresponding energy consumption. Multiplication of this value by 100 allowed the values of efficiency to be expressed as percentages.

SUBJECTS

The ages, body surface areas and body weights of the subjects are presented in Table I. With a single exception (C.B.), all of the subjects were men.

Group I—Normal subjects. Four members of the laboratory staff and three patients with minimal apical tuberculosis served as controls. The three patients were asymptomatic and had been ambulatory for several months prior to the time when the tests were performed.

Group II—Patients with chronic pulmonary emphysema. This group was composed of six patients who exhibited the clinical, roentgenologic and physiologic signs of moderate or advanced chronic pulmonary emphysema, but who had no evidence of cysts. The results of the pulmonary function tests, presented in Table II, indicated that each of the patients had a smaller than normal maximum breathing capacity and a larger than normal ratio of residual volume to total lung capacity. Four patients had arterial blood oxygen unsaturation at rest, and two of these four had elevated blood tensions of carbon dioxide.

Group III—Obese patients. Five obese patients who complained of dyspnea on exertion were included in this group. The results of the pulmonary function tests and the arterial blood studies are shown in Table II. Patient A.H. presumably suffered from arteriosclerotic heart disease and had been in congestive heart failure one year previously. At the time of the study he had been maintained satisfactorily for nine months on a regimen which included a low salt diet, weekly injections of Mercuhydrin® (meralluride) and a daily dose of digitalis.

RESULTS

The results are presented in Tables III, IV and V. The tabulated numbers were obtained from curves such as those drawn in Figure 1.

a) Energy used for breathing

The total energy used for resting ventilation by the normal subjects varied between 0.8 and 1.9 Kg. M. per L. per minute, while that used by the patients with emphysema varied between 2.1 and 8.5 Kg. M. per L. per minute. These observations agree with those previously made in this laboratory (1). The values recorded in the obese patients were larger: They ranged between 6.6 and 26.2 Kg. M. per L. per minute.

The energy used for ventilation during voluntary hyperpnea showed considerable individual

TABLE III

Values of ventilation, energy, work and efficiency recorded in the seven normal subjects in Group I

Subject	Ventilation	Energy cost of breathing	Mechanical work performed on lungs	Efficiency
	L./min./M.²	*Kg.M./min./M.²*	*Kg.M./min./M.²*	%
G.P.	5	5	0.1	2
	10	21	0.3	1.4
	15	44	0.6	1.4
	20	75	1.0	1.3
H.F.	7	6	0.4	6.7
	10	9	0.5	5.6
	15	19	0.8	4.2
	20	36	1.1	3.1
	25	61	1.8	3.0
	30	95	2.5	2.6
R.B.	5	6	0.2	3.3
	10	14	0.4	2.9
	15	26	0.7	2.7
	20	51	1.0	2.0
	25	90	1.6	1.8
	30	137	2.7	2.0
	35	189	2.4	2.2
J.F.	5	9	0.2	2.2
	10	23	0.4	1.7
	15	36	0.6	1.7
	20	74	0.9	1.2
	25	99	1.3	1.3
C.B.	10	9	0.5	5.6
	12	11	0.7	6.4
	14	17	0.8	4.7
	16	24	1.0	4.2
	18	33	1.2	3.6
	20	48	1.5	3.1
A.S.	3	6	0.2	3.3
	5	10	0.3	3.0
	7	23	0.4	1.7
	9	31	0.5	1.6
	11	45	0.6	1.3
	13	60	0.8	1.3
	15	78	0.9	1.2
	17	97	1.2	1.2
A.D.	10	18	0.6	3.3
	15	27	1.0	3.7
	20	50	1.7	3.4
	25	100	2.5	2.5
	30	176	3.5	2.0
	35	250	4.6	1.8

variation. In general, the energy expenditure at a particular level of ventilation was larger in the patients with emphysema and in the obese patients than it was in the subjects in the control group.

b) Mechanical work performed on the lungs

The values of work measured in the normal subjects were slightly larger than those reported by others. This is probably attributable to the imposed frequency of 30, rather than the lower, spontaneous frequencies which the subjects might naturally have chosen.

In accord with previous observations, the work required for any particular minute volume was larger in the patients with emphysema than in the normal subjects. Although the values were possibly different at the frequency of 30 than they would have been for the same minute volume respired at a lower frequency, the calculated amounts of work correspond to those previously reported for patients with disease of comparable severity.

The work performed on the lungs was abnormally high in only two of the obese patients. In the remaining three, the values were of the same order of magnitude as those recorded for the subjects in the control group.

c) Efficiency

The values of efficiency calculated for the normal subjects are plotted in Figure 3. In general, the efficiencies were maximal during resting venti-

TABLE IV

Values of ventilation, energy, work and efficiency recorded in the six patients with chronic pulmonary emphysema in Group II

Subject	Ventilation	Energy cost of breathing	Mechanical work performed on lungs	Efficiency
	L./min./M.²	*Kg.M./min./M.²*	*Kg.M./min./M.²*	%
T.B.	8	48	0.7	1.5
	12	74	0.9	1.2
	16	118	1.3	1.1
	18	146	1.5	1.0
	22	211	2.4	1.1
L.C.	6	56	1.2	2.1
	8	71	1.6	2.3
	10	109	2.6	2.4
W.L.	10	48	1.2	2.5
	12	112	1.8	1.6
	14	162	2.4	1.5
E.E.	7	54	0.5	0.9
	9	70	0.8	1.1
	10	107	1.1	1.0
	13	146	1.6	1.1
D.G.	8	19	0.4	2.1
	14	33	0.9	2.7
	19	52	1.8	3.1
	25	90	2.8	3.1
	30	120	4.3	3.6
J.G.	9	44	0.8	1.8
	12	113	1.2	1.1
	14	141	1.6	1.1

TABLE V

Values of ventilation, energy, work and efficiency recorded in the five obese patients in Group III

Subject	Ventilation	Energy cost of breathing	Mechanical work performed on lungs	Efficiency
	L./min./M.²	*Kg.M./min./M.²*	*Kg.M./min./M.²*	%
H.W.	5	43	0.3	0.7
	7	60	0.5	0.8
	9	79	0.7	0.9
	11	103	0.9	0.9
	13	131	1.2	0.9
	15	159	1.5	0.9
G.W.	5	130	0.6	0.5
	8	210	1.0	0.5
	10	261	1.4	0.5
	12	320	2.3	0.7
J.B.	6	87	1.2	1.4
	8	118	1.5	1.3
	10	155	1.8	1.2
	12	184	2.1	1.1
	14	202	2.5	1.2
M.S.	6	39	0.8	2.1
	10	68	1.1	1.6
	15	126	1.6	1.3
	20	191	2.8	1.5
	25	269	4.5	1.7
A.H.	5	69	0.3	0.4
	7	95	0.6	0.6
	9	124	0.9	0.7
	11	150	1.3	0.9

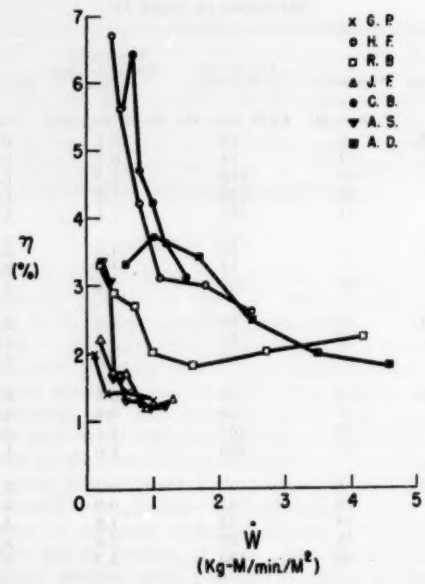

FIG. 3. RELATION BETWEEN EFFICIENCY (η) AND THE MECHANICAL WORK PERFORMED ON THE LUNGS (Ẇ) IN THE SEVEN NORMAL SUBJECTS

lation, then dropped progressively as the minute volume of ventilation was increased. The highest values were recorded in Subjects H.F. and C.B., each of whom realized an efficiency between 6 and 7 per cent.

The values of efficiency calculated for the patients with emphysema are compared to those of the normal subjects in Figure 4. Using the work performed per square meter of surface area as a reference, we found that three of the patients realized efficiencies comparable to those of the normal subjects, while the remaining three had efficiencies at the lower end of the normal range. These observations suggest that the chests of the patients with emphysema performed a given amount of work on the lungs with efficiencies not greatly different from normal. It is important to recognize, however, that this amount of work represents a large swing in pressure for a small change in volume. Hence, for any particular amount of work, the patients with emphysema derived a much smaller tidal exchange.

As can be see in Figure 5, the efficiencies of the

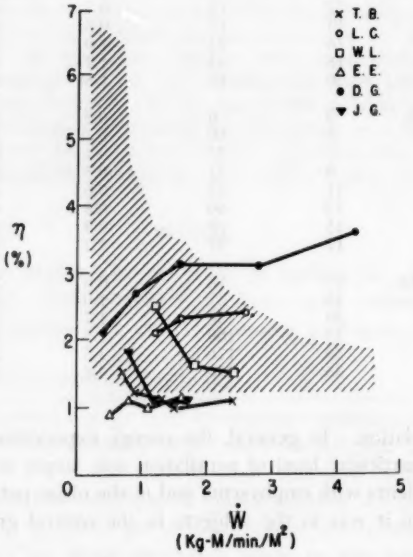

FIG. 4. RELATION BETWEEN EFFICIENCY (η) AND THE MECHANICAL WORK PERFORMED ON THE LUNGS (Ẇ) IN THE SIX PATIENTS WITH CHRONIC PULMONARY EMPHYSEMA

The hatched area defines the locus of the curves of the normal subjects.

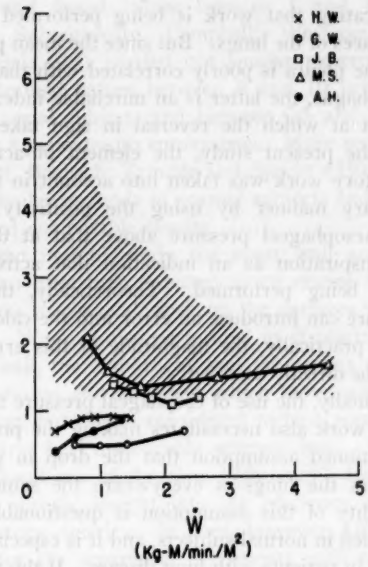

FIG. 5. RELATION BETWEEN EFFICIENCY (η) AND THE MECHANICAL WORK PERFORMED ON THE LUNGS (W) IN THE FIVE OBESE PATIENTS

The hatched area defines the locus of the curves of the normal subjects.

obese patients at all work loads tended to be lower than those found either in the normal subjects or in the patients with emphysema. Since the mechanical work performed on the lungs was either normal or only moderately increased, the reduction in efficiency was due to an abnormally high energy cost.

DISCUSSION

A) Limitations of the methods used to estimate energy and work

Liljestrand's method for estimating the oxygen cost of breathing (2) is based on the assumption that the extra oxygen used during augmented ventilation primarily reflects the extra oxygen utilized by the respiratory muscles in amplifying the excursions of the chest. This assumption seems reasonable, and the application of the principle has provided considerable information about the energetics of ventilation in both health and disease. However, the accuracy of the method is difficult to evaluate. For instance, the curves drawn in Figure 1, A represent visual averages of points that show considerable scatter, and near the resting level this scatter can markedly affect the estimates of both the change in the oxygen used for breathing and the total oxygen cost. During augmented ventilation, the scatter constitutes a smaller fraction of the respiratory component, and the oxygen used for breathing can be calculated with more confidence.

Comparable difficulties attend the calculation of the mechanical work performed on the lungs. This calculation is based on the concept that such work is done whenever the application of a pressure difference across a body produces a volume change. Hence,

$$W = \int P\,dV, \qquad 1)$$

where W = mechanical work, P = pressure difference, and dV = incremental volume change.

When this relation is used to calculate the work performed on the lungs, two features must be recognized: *1*) P is the difference in pressure existing between the mouth and the pleura; and *2*) the use of a single value of P, such as that measured between the mouth and a point on the pleural surface, necessitates assuming that the pressure difference across the lungs is everywhere the same. Both of these features assume importance whenever the pressure in the esophagus is substituted for that in the pleura. Because the mean pressures in the two spaces are not well correlated (9, 10), pressure-volume diagrams must

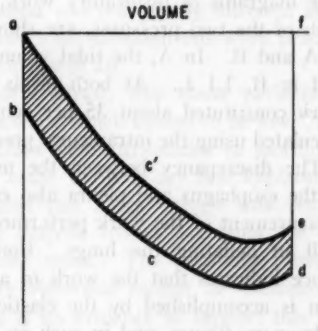

FIG. 6. SCHEMATIC PRESSURE-VOLUME DIAGRAM DEPICTING THE THEORETICAL EFFECT OF ASSUMING THAT THE DIFFERENCE IN PRESSURE ACROSS THE LUNGS AT THE BEGINNING OF INSPIRATION IS ZERO

For explanation, see text.

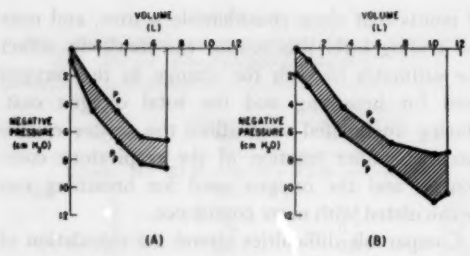

FIG. 7. COMPARISON OF PRESSURE-VOLUME DIAGRAMS PLOTTED USING INTRAESOPHAGEAL PRESSURE (P_e) AND INTRAPLEURAL PRESSURE (P_P)

In (A), the tidal volume was 0.7 L., and in (B), 1.1 L.

be plotted on the assumption that the pressure difference across the lungs at the start of inspiration is zero (10–14). The validity of this assumption is questionable, and its theoretical effect is illustrated in Figure 6. In the drawing, the pressure-difference between the mouth and the pleura at the start of inspiration is indicated by the vertical distance *ab*, and the work of inspiration is represented by the area *abcdefa*. If, when using the esophageal pressure, *ab* is assumed to be zero, then the work of inspiration is represented by the area *ac'efa*. In the latter instance, the work delineated by the hatched area is neglected, and the calculated work is less than that actually performed. To investigate the size of this error, we obtained simultaneous records of the change in the volume of the lungs, the intraesophageal pressure and the intrapleural pressure in one of the patients with pneumothorax. The diagrams of inspiratory work, plotted using each of the two pressures, are shown in Figures 7, A and B. In A, the tidal volume was 0.7 L., and in B, 1.1 L. At both levels the neglected work constituted about 35 per cent of the work calculated using the intrapleural pressure.

The discrepancy between the mean pressures in the esophagus and pleura also complicates the measurement of the work performed by the chest wall in deflating the lungs. Considerable evidence indicates that the work of a quiet expiration is accomplished by the elastic recoil of the pulmonary tissues, and in such an expiration the intrapleural pressure should remain less positive than the pressure of air at the mouth. By way of contrast, a forced expiration or an expiration against resistance can produce such positivity, indicating that work is being performed on the surfaces of the lungs. But since the mean pressure in the pleura is poorly correlated with that in the esophagus, the latter is an unreliable index of the point at which the reversal in sign takes place. In the present study, the element of active expiratory work was taken into account in the customary manner by using the positivity of the intraesophageal pressure above that at the start of inspiration as an indication that active work was being performed. Theoretically, this procedure can introduce an error into the calculation, and practically, the magnitude of this error cannot be directly assessed.

Finally, the use of esophageal pressure to calculate work also necessitates making the previously mentioned assumption that the drop in pressure across the lungs is everywhere the same. The validity of this assumption is questionable when applied in normal subjects, and it is especially suspect in patients with lung disease. If the pressure were different at different points on the lung surface, the calculation of work would necessitate first measuring the pressure and the volume change at each point on the pleura, and then substituting these values into Equation *2*).

$$W = \int P_1 dV_1 + \int P_2 dV_2 + \cdots \int P_n dV_n. \quad 2)$$

Such measurements are not feasible, and if they could be made, the calculation of work would be discouragingly complex.

For all of the aforementioned reasons, we believe that the use of esophageal pressure for estimating the work performed on the lungs may introduce errors of considerable magnitude, and we share the opinion of others that *increments* of work, rather than *absolute* values, may have the most validity.

B) Efficiency with which the work on the lungs is accomplished

The efficiency of the ventilatory apparatus has been considered in six previous publications. Rahn and his co-workers (15), Otis, Fenn and Rahn (16) and Otis alone (11) obtained values in normal subjects of 5 per cent or less. Cain and Otis (17) calculated the efficiency with which normal subjects performed work on external resist-

ances and found values between 1 and 4 per cent. Subsequently, Campbell, Westlake and Cherniack (18) carried out similar experiments and obtained values between 8 and 10 per cent in a normal subject, and between 0.5 and 2 per cent in a patient with emphysema. More recently, Bartlett, Brubach, Trimble and Specht (19) have used similar methods in normal subjects and have obtained comparable results.

In the present study the chest wall and diaphragm have been depicted as an engine which performs mechanical work on the surfaces of the lungs. Work is also performed in displacing the chest wall, the diaphragm, and the organs of the mediastinum and abdomen which move with respiration, but this is considered to be a source of necessary but undesirable energy loss. Thus, the present use of the term "efficiency" does not afford specific insight into the efficiency of the respiratory muscles. It does, however, afford a basis for the separation of patients with a high energy cost of breathing into two groups: *1*) those in whom the increased cost is attributable to the lungs, and *2*) others in whom it is not.

According to this concept, our data suggest that although a patient with emphysema is forced to perform abnormally large amounts of work to mobilize his lungs, this work is performed with an efficiency that is not greatly different from that realized by a normal person at a comparable work load. However, the two types of subjects have the important difference mentioned previously: For a particular amount of work performed on the lungs, the patient with emphysema derives a much smaller exchange of air.

The values of efficiency calculated for the obese patients tended to be low. Two of these patients (H.W. and J.B.) suffered from the effects of alveolar hypoventilation as indicated by high tensions of carbon dioxide in the arterial blood. Both patients expended inordinately large amounts of energy for breathing, despite the fact that the work performed on their lungs was not increased. These data can be explained by invoking either of two mechanisms: *1*) The excess energy was used to impart motion to extrapulmonary structures; or *2*) the muscles of the obese patients consumed excessive energy in performing a given amount of mechanical work. The first of these possibilities seems more likely because these patients must have performed extra work in moving the adipose tissues overlying the chest and abdominal walls. The second possibility cannot be eliminated, although there is no evidence that the muscles of an obese person behave in an abnormal way.

Finally, it was emphasized previously that appreciable errors may attend the measurements of *total* energy and *total* work, and for this reason it is inappropriate to place too much emphasis on the calculated values of efficiency. However, changes in energy and work can be calculated with more assurance, and we compared the patients with emphysema and the obese patients in a second way. This second comparison entailed calculating the change in energy required to increase the work from 1.2 to 1.5 Kg. M. per minute per $M.^2$. This particular increment in work was chosen because, with the exception of A.H., all of the patients in the two groups performed work in this range. The average value for the six patients with emphysema was 21 Kg. M. per minute per $M.^2$, with a range of 5 to 31. The average value for the four obese patients was 41 Kg. M. per minute per $M.^2$, with a range of 21 to 57. Hence, the data support the view that the obese patients expended excessive amounts of energy which could not be attributed to the work done on the lungs.

SUMMARY

The efficiency of the chest wall and diaphragm of seven normal subjects was less than 7 per cent. The efficiency of these structures in six patients with emphysema was not greatly different at any particular work load, although this work produced a smaller exchange of air.

By way of contrast, five obese patients had values of efficiency which tended to be low. Since these low values were attributable to inordinately high energy costs, the data are best explained by presuming that this extra energy was required to move the adipose tissues overlying the chest and abdominal walls. The possibility that the muscles were abnormal cannot be excluded, although there is no evidence to support this view. Whatever the reason for the extra energy consumption, its expenditure presumably contributed to the dyspnea these patients experienced on exercise.

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COMBINED DRUG TREATMENT OF TUBERCULOSIS. I. PREVENTION OF EMERGENCE OF MUTANT POPULATIONS OF TUBERCLE BACILLI RESISTANT TO BOTH STREPTOMYCIN AND ISONIAZID *IN VITRO*

By MAURICE L. COHN, GARDNER MIDDLEBROOK AND WILLIAM F. RUSSELL, JR.

(From the Department of Research and Laboratories, National Jewish Hospital at Denver, and the Departments of Medicine and Microbiology, University of Colorado School of Medicine, Denver, Colo.)

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Treatment of tuberculosis with single drug regimens employing streptomycin, para-aminosalicylic acid, or isoniazid has proved to be clinically inferior to the use of combinations of these drugs (1–3). The superiority of combined drug regimens has been manifest both in the percentage of patients rendered bacteriologically negative and in the lower incidence of the emergence of drug resistance.

Ehrlich (4) first conceived the generalization that chemotherapy combining two or more effective agents with independent actions would prove superior to single drug treatment by tending to prevent the parasite from developing resistance to the action of either of the drugs. This principle was applied to antimicrobial attack *in vitro* on *Mycobacterium tuberculosis* by Middlebrook and Yegian (5), who also outlined the theoretical basis for prevention of the emergence of drug-resistant mutants of tubercle bacilli by combined drug treatment. Demerec (6) employed more refined genetic principles to elaborate upon this theory. Then Karlson and co-workers (7) demonstrated the suppressive effect of combined drugs (streptomycin, para-aminosalicylic acid and promin) upon the emergence of streptomycin-resistant tubercle bacilli during the chemotherapy of tuberculosis in man. Since that time many studies pertaining to combined drug therapy have been reported both from the laboratory and from clinical investigation.

Thus, although it has been impossible to provide clinical evidence for superiority of combined drug treatment in many other infectious diseases, there seems to be no doubt of its superiority over the use of single drugs in cavitary pulmonary or renal tuberculosis. Nevertheless, in spite of this established superiority of combined drug regimens, the sputa of 10 to 20 per cent of previously untreated patients fail to become bacteriologically negative, on conventional double-drug programs, without ancillary collapse procedures or surgical intervention (8–10). Many of these chemotherapeutic failures are the result of the emergence of mutant forms [1] of tubercle bacilli which are resistant to one or more of the antimicrobial agents employed (12, 13).

Clinical investigation and laboratory studies have established the fact that the action of a single antimicrobial agent in adequate concentration *in vitro* will usually select a drug-resistant population of tubercle bacilli if, initially, the number of organisms is sufficiently large, and if they multiply to yield a detectable colony. Such investigations depend upon laboratory determination of the prevalence of drug-resistant mutants in large populations of the parasites. Most attempts to estimate the prevalence of drug-resistant mutants have been performed with cultures containing relatively small numbers of organisms, in liquid media, and with techniques requiring repeated subculture.

[1] The advisability of employing the term *mutants* to describe typical tubercle bacilli which are found to be resistant to the antimicrobial action of a drug may be questioned. This usage has been justified on the following grounds: *1)* Such organisms immediately proliferate from previously unexposed populations of organisms just as though they were present in such populations prior to selection by the suppressive action of the drug on the susceptible organisms. *2)* All other peculiarities of their properties are just as immediately apparent. *3)* In the case of one strain of tubercle bacilli this has been proved by replica plating techniques which allow for precise estimation of the drug susceptibility of clones before any drug action (11).

Such techniques are not adequate for a real estimation of the prevalence of drug-resistant mutants; only a few studies (14, 15) have been performed which provide the quantitative information needed.

In order to provide a rational bacterial genetic basis for the superiority of combined drug regimens and, perhaps, also for the not uncommon failure of this approach to chemotherapy of tuberculosis with conventional drug dosages, quantitative information is required on the prevalence of mutants resistant to two drugs (double mutants) in wild, previously unexposed populations of tubercle bacilli. Whether or not, and, if so, how many mutant forms simultaneously resistant to both streptomycin and isoniazid can be selected *in vitro* from extremely large populations of tubercle bacilli exposed to both of these agents in combination obviously has important clinical implications. The purpose of this report (the first of a series) is to present the results of a study of this problem.

MATERIALS AND METHODS

1. Culture medium. The experiments were performed with base medium 7H-10,[2] the composition and preparation of which follows:

KH_2PO_4 (anhydrous)	1.5	Gm.
Na_2HPO_4 (anhydrous)	1.5	Gm.
L-glutamic acid	0.5	Gm.
Ammonium sulfate	0.5	Gm.
Glycerol (reagent grade)	5.0	ml.
Tri-sodium citrate $2H_2O$	0.4	Gm.
Ferric ammonium citrate	0.04	Gm.
$MgSO_4 \cdot 7H_2O$	0.05	Gm.
$CaCl_2 \cdot 2H_2O$	0.0005	Gm.
$ZnSO_4 \cdot 7H_2O$	0.001	Gm.
$CuSO_4 \cdot 5H_2O$	0.001	Gm.
Pyridoxine hydrochloride	0.001	Gm.
Biotin	0.005	Gm.

Dissolve the above in 1,000 ml. of distilled water, and adjust to pH 6.6. For growth on solid medium, add agar [3] in a final concentration of 1.5 per cent and add 0.25 mg. malachite green (oxalate salt) per ml. before autoclaving. After autoclaving, add oleic acid-albumin complex (16) in a final concentration of 0.5 per cent. For growth in liquid ST (standard transfer) medium, before autoclaving add Tween® 80 in a final concentration of 0.05 per cent, and add after autoclaving albumin instead of oleic acid-albumin complex, and omit agar. In either case, after autoclaving, add 0.2 per cent final concentration of glucose (from a concentrated solution previously autoclaved with 0.005 M citric acid), and catalase [4] in final concentration of 3 μg. per ml. from a stock solution, 1.0 mg. per ml. in 0.85 per cent saline, sterilized by passage through a sintered-glass filter.

2. Drugs. Sterile streptomycin sulfate powder in rubber-stopper bottles was dissolved aseptically in sterile distilled water to achieve a final concentration of 1,000 μg. of the base per ml. This stock solution was stored at − 10° C. Isoniazid was dissolved in sterile distilled water to a final concentration of 1,000 μg. per ml. This solution was then autoclaved for 10 minutes at 15 pounds pressure (17), and stored at 4° C.

Petri dishes (100 mm. diameter) were prepared with the oleic acid-albumin-agar medium, designated 7H-10. The drugs were added aseptically to this sterile medium just prior to pouring. In the first experiment, four plates were prepared with 1.0 μg. of isoniazid per ml. of solid culture medium, four plates contained 2.0 μg. of streptomycin per ml., and 100 plates were poured with both 2.0 μg. of streptomycin and 1.0 μg. of isoniazid per ml. For the second experiment two plates were prepared containing 1.0 μg. of isoniazid per ml., two plates were prepared containing 1.0 μg. of streptomycin per ml., and 100 plates containing both 1.0 μg. of streptomycin and 1.0 μg. of isoniazid per ml. were poured. The third experiment was the same as the first except that 0.2 μg. of isoniazid instead of 1.0 μg. per ml. was used both in the plates with isoniazid alone and in the 100 plates testing the combined action of streptomycin and isoniazid. The fourth experiment was carried out in liquid medium (ST); it was designed to reveal the sterilizing rather than the bacteriostatic action of 2.0 μg. of streptomycin and 0.5 μg. of isoniazid per ml. of liquid culture medium. These concentrations of drugs were selected because such concentrations have been achieved in the serum of patients during treatment.

3. Cultures. Fully grown laboratory strains of tubercle bacilli (H37Rv and Pearson I), cultivated in Tween®-albumin liquid medium, 7H-4, were used (18). H37Rv was used in both the first and second experiments. However, the stock H37Rv strain has yielded catalase-positive,[5] isoniazid-resistant mutants in our laboratory only in very small numbers. This differs from our experience with most "wild" strains of tubercle bacilli freshly recovered from patients, such as the Pearson I strain. Studies *in vitro* on "wild" strains have demonstrated (19) that, by and large, critically low concentrations of isoniazid select many weakly isoniazid-resistant mutants which retain significant degrees of catalase activity (called catalase-positive) while higher concentrations of isoniazid result in the selection of only catalase-negative, highly isoniazid-resistant forms. Thus, an approximate estimate of the concentration of isoniazid in the free active state can be made after isoniazid-resistant

[2] A similar medium, 7H-9, is available from Difco Laboratories, Detroit, Mich.

[3] Baltimore Biological Laboratories, Inc., Baltimore, Md.

[4] Nutritional Biochemical Corp., Cleveland, Ohio (listed as catalase "crude").

[5] Catalase testing as performed in our laboratory is a crude test. A drop or two of a cold aqueous solution 15 per cent hydrogen peroxide and 5 per cent Tween® is placed on the colony of tubercle bacilli. Catalase-negative organisms give little or no foaming. Catalase-positive organisms yield foaming promptly.

mutants have been selected; low concentrations sterilize only the susceptible parent organisms while higher concentrations sterilize all but the highly isoniazid-resistant, catalase-negative mutants. Because the standard laboratory strain, H37Rv, fails to yield catalase-positive, isoniazid-resistant mutants, and, therefore, does not behave in the usual fashion toward high and low antimicrobially-active concentrations of isoniazid, the Pearson I strain which does yield both catalase-negative and catalase-positive mutants was employed in the third and fourth experiments.

4. Procedures. The first three experiments were performed in the same manner. Petri dishes containing only isoniazid were inoculated with 0.1 ml. of a 1×10^{-1} dilution of a seven to 10 day old dispersed culture of tubercle bacilli (neither filtered nor ground). These stock cultures in liquid medium have been shown to contain approximately 5×10^8 viable bacterial units per ml. (20). Distribution of the organisms on each plate was accomplished by means of a spreader while the plate was rotating on a turntable. Similarly, the plates containing streptomycin alone were inoculated with 0.1 ml. of the undiluted suspension, and the plates which contained both streptomycin and isoniazid were inoculated each with 0.2 ml. of the undiluted suspension. All of the plates were incubated at 36° C. under an atmosphere of 2 to 5 per cent carbon dioxide in air for at least 28 days. Drying out of the cultures was prevented by enclosing the plates in polyethylene plastic bags.

The fourth experiment was performed in duplicate. Two 250 ml. flasks containing 20 ml. of the liquid medium (ST) were inoculated with 0.2 ml. of an undiluted suspension of five day old vigorously growing Pearson I organisms. These flasks were incubated in the same way as the plates; however, two 0.1 ml. samples were removed from each duplicate flask at zero time, one, two and three weeks, and were plated on Petri dishes containing the 7H-10 solid medium without added drugs. These plates were incubated as indicated above.

RESULTS

Experiment No. 1

Four plates were used to test 1.0 μg. of isoniazid per ml. alone; four plates for 2.0 μg. of streptomycin alone; and 100 plates tested 2.0 μg. of streptomycin combined with 1.0 μg. of isoniazid (Table I). The 108 plates were incubated as described under Materials and Methods, and then were examined. The isoniazid plates yielded 99, 101, 106 and 107 colonies, an average of 103 colonies, consisting of isoniazid-resistant mutants. It is assumed that each of these colonies arose from a single drug-resistant mutant because of the relatively small numbers of organisms in each clump. Since there were roughly 5×10^6 viable bacterial units originally inoculated onto each isoniazid plate, it is evident that there was approximately one isoniazid-resistant mutant for every 5×10^4 drug-susceptible bacterial units. This is in agreement with previous estimates of the prevalence of isoniazid-resistant mutants in the H37Rv strain (15).

TABLE I

Results of Experiment 1

Number of plates	Inoculum* per plate	Drug conc.	Colonies per plate (actual)	Prevalence of resistance mutants
		μg./ml.		
4	5×10^6	INH† 1.0	99, 101, 106, 107	1 in 5×10^4
4	5×10^7	SM‡ 2.0	55, 59, 64, 70	1 in 1×10^6
100	1×10^8	INH 1.0 SM 2.0	No growth	<1 in 1×10^{10}

* Number of viable, bacterial units from a seven to 10 day old vigorously growing, well dispersed *H37Rv* culture in liquid medium (ST).
† Isoniazid.
‡ Streptomycin.

The streptomycin plates supported growth of 55, 59, 64 and 70 colonies, an average of 62 colonies, consisting of streptomycin-resistant mutants. This represents about one streptomycin-resistant mutant in 1×10^6.

The 100 plates testing the combined action of streptomycin and isoniazid yielded no growth whatsoever. In other words, no mutant which was simultaneously resistant to both streptomycin and isoniazid, the so-called double mutant, was present in 1×10^{10} viable bacterial units of the H37Rv strain. This population is about one-tenth of the number theoretically necessary to yield one such double mutant.

Only isoniazid-resistant mutants were recovered from the colonies present on those plates which contained isoniazid alone; and only streptomycin-resistant mutants were present in the colonies which grew on the plates containing 2.0 μg. of streptomycin per ml. of solid culture medium. The combination of 2.0 μg. of streptomycin and 1.0 μg. of isoniazid per ml. of culture medium completely prevented the appearance of any colonies.

Experiment No. 2

This experiment was the same as the first except that only 1.0 μg. of streptomycin per ml. was

TABLE II

Results of Experiment 2

Number of plates	Inoculum* per plate	Drug conc.	Colonies per plate (actual)	Prevalence of resistant mutants
		μg./ml.		
2	2.5×10^6	INH 1.0	23, 26	1 in 1×10^5
2	2.5×10^7	SM 1.0	30, 30	1 in 8×10^5
100	5×10^7	INH 1.0 SM 1.0	10†	1 in 5×10^8

* Number of viable bacterial units from a seven to 10 day old vigorously growing, well dispersed *H37Rv* culture in liquid medium (ST).

† Only 10 colonies appeared on a total of 100 plates; all susceptible to SM (streptomycin), 0.5 μg. per ml.; resistant to INH (isoniazid), catalase-negative.

employed (Table II). After the 104 plates had been incubated for 28 days, they were inspected for evidence of growth. The isoniazid plates revealed 23 and 26 large colonies, all of which proved to consist of isoniazid-resistant, catalase-negative organisms. There was one isoniazid-resistant mutant in 1×10^5 viable bacterial units. The streptomycin plates revealed a thin, diffuse spreading growth of streptomycin-susceptible organisms over the entire surface of each plate. These organisms had been prevented from proliferating during only part of the incubation period. A total of 60 large colonies were recognizable on these plates. These represented organisms which were streptomycin-resistant. Roughly speaking, this showed that among 8×10^5 drug-susceptible bacterial units there was one streptomycin-resistant mutant.

The 100 plates testing the combined action of streptomycin and isoniazid revealed 10 small colonies. These colonies proved on subculture to consist of pure populations of organisms which were susceptible to 0.5 μg. of streptomycin per ml. of culture medium and resistant to at least 0.5 μg. of isoniazid. Since approximately 5×10^9 viable bacterial units were seeded on these plates, only one isoniazid-resistant mutant in 5×10^8 drug-susceptible bacterial units was able to proliferate on medium containing the stated concentrations of both drugs together.

This experiment emphasized the fact that population size significantly modifies the antimicrobial action of streptomycin. A concentration of about 0.2 μg. of streptomycin per ml. of solid culture medium is usually inhibitory, if not bactericidal, for small numbers of tubercle bacilli of the H37Rv strain. These results show that when large numbers of tubercle bacilli are used, even a fivefold increase to 1.0 μg. of streptomycin per ml. is not completely inhibitory for all drug-susceptible organisms.

The usual minimum antibacterial concentration of isoniazid for small numbers of tubercle bacilli of the H37Rv strain is 0.05 to 0.075 μg. per ml. No significant antagonistic effect attributable to large inocula of tubercle bacilli has been observed in the case of isoniazid. The almost, but not quite, adequate concentration of streptomycin was certainly responsible for the marked reduction in numbers of isoniazid-resistant colonies which did grow. These experiments provide evidence for cross-antibacterial activity between streptomycin and isoniazid. However, when combined drug activity failed due to an inadequate concentration of Drug "A" (in this case, streptomycin), mutants grew which were resistant to Drug "B" (isoniazid) which was present in more effective concentration.

Experiment No. 3

The previous experiments demonstrated that a concentration of 2.0 μg. of streptomycin was the minimum totally inhibitory concentration for such a large population of tubercle bacilli. On the other hand, 1.0 μg. of isoniazid was greatly in excess of the minimum antibacterial concentration of isoniazid for small numbers of tubercle bacilli of this strain, H37Rv.

Catalase-positive, isoniazid-resistant mutants can be derived from the H37Rv strain by antimicrobially-active concentrations of isoniazid only with great difficulty, although catalase-negative, isoniazid-resistant mutants are readily selected. When low concentrations of isoniazid were under investigation, an organism which could produce both types of resistant mutants was necessary to establish the fact that the concentration of isoniazid was suboptimal, even though exceeding the minimum antibacterial concentration for the drug-susceptible parent stock. In such an experiment a strain which readily yields a significant number of catalase-positive, isoniazid-resistant mutants when subjected to the action of critically low concentrations of isoniazid might reveal significantly different responses to the antimicrobial activity of the combination of both drugs. Therefore, be-

cause it readily yields both types of mutants, the Pearson I strain was used.

The Pearson I strain (streptomycin-susceptible, isoniazid-susceptible, catalase-positive, pathogenic) was seeded on 108 plates using the same dilutions as in the preceding experiments (Table III). After incubation as before, these plates were read. The isoniazid plates revealed 34, 44, 45 and 54 colonies, respectively, an average of 44 colonies, composed of isoniazid-resistant mutants. In accordance with the previous assumption, this means that there was roughly one isoniazid-resistant mutant among 1×10^5 drug-susceptible bacterial units. There were 39, 39, 40 and 44 colonies on the streptomycin plates, an average of 40 colonies consisting of streptomycin-resistant mutants, or about one streptomycin-resistant mutant in 1×10^6 drug-susceptible bacterial units. The 100 plates containing both 2.0 μg. of streptomycin and 0.2 μg. of isoniazid per ml. of solid medium yielded no growth.

The colonies which grew on the plates containing only isoniazid were investigated for catalase activity. On one plate all 54 colonies were subjected to catalase testing: 41 colonies gave a negative reaction, and 13 colonies gave a positive reaction. Six colonies were selected at random from another of the isoniazid plates in order to investigate isoniazid resistance as well as catalase activity. One colony consisted of organisms resistant to 0.2 μg. of isoniazid, but susceptible to 5.0 μg. These organisms were catalase-positive. The five remaining colonies proved to consist of pure

TABLE III

Results of Experiment 3

Number of plates	Inoculum* per plate	Drug conc.	Colonies per plate (actual)	Prevalence of resistant mutants
		μg./ml.		
4	5×10^6	INH 0.2	34, 44,† 45, 54‡	1 in 1×10^5
4	5×10^7	SM 2.0	39, 39, 40, 44	1 in 1×10^6
100	1×10^8	INH 0.2 SM 2.0	No growth	<1 in 1×10^{10}

* Number of viable bacterial units from a seven to 10 day old vigorously growing, well-dispersed *Pearson I* culture in liquid medium (ST).

† Six colonies selected at random: one catalase-positive, R-0.2 INH (isoniazid), S-5.0 INH; five catalase-negative, R-0.2 and 5.0 μg. INH.

‡ Fifty-four colonies: 41 catalase-negative, 13 catalase-positive.

populations which were catalase-negative and resistant to both 0.2 and 5.0 μg. of isoniazid per ml. of medium. These same colonies were susceptible to the antimicrobial action of 2.0 μg. of streptomycin per ml.

The results of this experiment confirmed our previous observations that exposure of large numbers of the Pearson I strain (total for the four isoniazid plates was 2×10^7 viable bacterial units) to 0.2 μg. of isoniazid per ml. selected both catalase-positive mutants which were resistant to 0.2 μg. of isoniazid and susceptible to 5.0 μg. of isoniazid per ml. of culture medium, and catalase-negative mutants which were resistant to both 0.2 and 5.0 μg. of isoniazid per ml.

Although this concentration of isoniazid was able to manifest effective antibacterial activity against all drug-susceptible parent bacilli, it was still low enough to select both types of resistant mutants. It seemed possible that a differential selection of the two types of resistant mutants might result under the influence of combined streptomycin and low isoniazid. No such differential selection occurred.

Experiment No. 4

The first three experiments demonstrated the antibacterial effect of combined drug action on very large populations of tubercle bacilli; this fourth experiment was designed to show whether this antibacterial activity was bacteriostatic or sterilizing. Viable colony counts were made by plating out from liquid culture medium (ST) containing drugs onto oleic acid-albumin-agar plates containing no drugs. Two duplicate flasks, each containing 0.5 μg. of isoniazid and 2.0 μg. of streptomycin per ml., were sampled in duplicate at zero time, and the samplings (each 0.1 ml.) plated on Petri dishes. These four control plates revealed confluent growth after 28 days' incubation. The flasks were sampled in duplicate after seven days of incubation. This time the plates revealed four, four, three and zero colonies. Sampling of the flasks at 14 and 21 days failed to yield any colonies, indicating a marked sterilizing effect upon the 2×10^8 viable bacterial units originally inoculated. This experiment confirms the observations of Singh and Mitchison (21) relating to the marked sterilizing activity of these two drugs for multiplying tubercle bacilli.

DISCUSSION

Replica plating techniques have established that isoniazid-resistant mutants of typical tubercle bacilli are present in large populations of isoniazid-susceptible organisms prior to any exposure to this drug (11). Presumably this is also true for streptomycin-resistant mutants. Therefore, whether or not these drugs also stimulate more rapid mutation to drug resistance is not a matter for further consideration in this report.

Analysis of the bacteriologic records of patients treated inadequately in the past with streptomycin and isoniazid revealed that the drug-resistant organisms which appeared during treatment were initially resistant to only one drug.[6] Theoretically, there is a definite but extremely low incidence of mutants simultaneously resistant to both of these drugs in enormous populations. Effective concentrations of both streptomycin and isoniazid should select such double mutants when they are present. It is evident that in our four experiments the populations tested were not sufficiently large to provide evidence on this point.

These experiments do establish by quantitative methods the phenomenon of cross-sterilization of multiplying single-drug-resistant mutants *in vitro* when streptomycin and isoniazid are employed, each in concentrations adequate to sterilize all multiplying parent drug-susceptible bacterial cells in "wild" populations of stock laboratory strains of H37Rv and Pearson I. Although the specific modes of action of these two drugs have not been biochemically defined, this evidence corroborates other observations that they have independent and unrelated mechanisms of action against drug-susceptible parasites.

These experiments also show that a nearly, but not quite, adequate concentration of streptomycin combined with relatively high concentrations of isoniazid reduces to a remarkable degree the survival of mutants resistant to isoniazid. Similarly, with an adequate concentration of streptomycin, a critically low concentration of isoniazid, alone unable to prevent the selection of weakly isoniazid-resistant, catalase-positive mutants, is quite sufficient to prevent the emergence of both isoniazid- and streptomycin-resistant mutants.

It should be emphasized that combined drug action, such as was tested here, actually *prevents* the emergence of drug resistance unless a spontaneous double mutant is encountered. These experiments failed to reveal even one double mutant from 2.5×10^{10} viable bacterial units.

Clinical experience indicated in the past that drug resistance would emerge provided only that the sputum remained persistently positive and combined drug therapy continued for a sufficient period of time. This led to the idea that combined drug treatment merely delayed the emergence of resistance. However, another interpretation of the emergence of drug resistance under these conditions (combined drug treatment and persistently positive sputum) is possible. This view is based on the results of Experiment No. 2, where an inadequate concentration of streptomycin allowed a few isoniazid-resistant mutants to be selected by fully adequate concentrations of isoniazid. According to this hypothesis, the patients who eventually excreted drug-resistant organisms had been unable to achieve or maintain for a sufficient period of time adequate concentrations of one of the two drugs in those sites where the tubercle bacilli were multiplying during the time that the other drug was being delivered in fully adequate concentration.

Finally, the results of these experiments provide a microbiologic explanation for the increased conversion rates in those clinical trials (8) which have employed lower dosage of isoniazid (3 to 5 mg. per Kg. per day) combined with higher dosage of streptomycin (7.0 Gm. or more per week). Five to 10 times higher concentrations of isoniazid (1.0 μg. per ml. as compared to 0.1 μg. per ml.) were required to prevent the *in vitro* emergence of catalase-positive, isoniazid-resistant mutants than were needed to exert a striking antibacterial effect against only the parent drug-susceptible population. Thus, in clinical practice, combined drug treatment employing lower dosages of isoniazid should be sufficient with adequate dos-

[6] It is possible that such drug-resistant organisms can be acquired from another patient by so-called *exogenous reinfection*. However, careful study of the records of the inadequately treated patients, referred to above, revealed not one case in which resistance appeared to a drug that the patient had not previously received. Therefore, these results are consistent with the generally accepted concept that the resistant forms of tubercle bacilli which emerge to predominance in the sputa of drug-treated patients are derived *in vivo* from spontaneous mutation in parent drug-susceptible populations.

age of streptomycin to prevent the multiplication of all mutants resistant to either drug (except, of course, double mutants).

SUMMARY

The antimicrobial activity of isoniazid combined with streptomycin was tested against large numbers of multiplying tubercle bacilli of the H37Rv and Pearson I strains in order to investigate the prevention of the selection of mutants simultaneously resistant to both drugs. Concentrations of streptomycin not quite sufficient alone to inhibit the growth of all streptomycin-susceptible bacterial cells were remarkably effective in combination with 20 times the minimum inhibitory concentration of isoniazid in reducing the emergence of drug-resistant mutants. Concentrations of isoniazid, whether only slightly or far in excess of the minimum inhibitory concentration, when combined with the minimum sterilizing concentration of streptomycin prevented the emergence of any drug-resistant mutants. Prevention of emergence of drug-resistant mutants was attributed to the cross-sterilizing action of the two drugs on individual mutants separately resistant to each one. The implications of these observations for clinical chemotherapy were discussed.

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COMBINED DRUG TREATMENT OF TUBERCULOSIS. II. STUDIES OF ANTIMICROBIALLY-ACTIVE ISONIAZID AND STREPTOMYCIN SERUM LEVELS IN ADULT TUBERCULOUS PATIENTS

By WILLIAM MANDEL, ANGELINE D. HEATON, WILLIAM F. RUSSELL, JR. AND GARDNER MIDDLEBROOK

(From the Department of Research and Laboratories, National Jewish Hospital at Denver, and the Departments of Medicine and Microbiology, University of Colorado School of Medicine, Denver, Colo.)

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The importance of achieving concentrations of both streptomycin and isoniazid adequate to sterilize all parent drug-susceptible organisms was empasized by *in vitro* studies on the prevention of emergence of mutants resistant to these drugs (1). Many factors determine the concentrations of these agents to which multiplying tubercle bacilli are exposed in the human subject: dosage; absorption and excretion; metabolic alteration; binding to protein; and diffusion into lesions, tissues and cells. Furthermore, with streptomycin the problem is complicated by the presence of substances in the lesions which antagonize the antibacterial action of the drug.

Metabolic alteration of isoniazid has been estimated from serum levels of antimicrobially-active drug collected at intervals following a test dose (2, 3). Isoniazid is altered to derivatives such as acetyl-isoniazid and isonicotinic acid which have little or no antimicrobial activity (4, 5). Bönicke and Reif (6), Cuthbertson, Ireland, Wolff and Kuper (7), and Hughes, Schmidt and Biehl (8) have found that the rate of metabolic alteration of the isoniazid molecule varies widely in different individuals. Mandel, Cohn, Russell and Middlebrook (9) demonstrated a significant relationship between this rate of metabolic alteration of isoniazid and the bacteriologic response in isoniazid-treated tuberculous patients. The chemical methods thus far described for the determination of isoniazid (10–14) are not practical for the antimicrobially-active moiety, as they are neither sufficiently sensitive nor specific to permit measurement of the low concentrations present in the sera of many subjects receiving conventional dosages of this drug.

Streptomycin serum concentrations have been investigated, and apparently this drug is not subject to significant metabolic alteration (15). There were differences between the serum concentrations of streptomycin achieved by four different subjects (16). Most studies have tended to emphasize the uniformity of the handling of streptomycin from subject to subject (17–20). However, very few human subjects were used.

The purpose of this report (the second of a series) is to present our experiences with specific microbiologic assays for isoniazid and streptomycin performed on sera from isoniazid- and streptomycin-treated adult tuberculous patients, and to discuss the clinical significance of the results.

MATERIALS AND METHODS

Microbiologic assay for serum isoniazid was performed as previously described (2). Isoniazid was given in total dosage of 8 to 16 mg. per Kg. daily for at least one day prior to the test. The initial study in most patients consisted of an assay on a blood specimen obtained by venipuncture at six hours after a 4 mg. per Kg. oral test or loading dose of isoniazid (given at 9:00 a.m.). Streptomycin (20 mg. per Kg.) was uniformly administered along with isoniazid on this first test day. The patients were not fasting, but this has not significantly modified the results of this assay as performed in our laboratory (2). With the exception of insulin in the case of diabetics, no other drugs were given on the test day. Para-aminosalicylic acid (NaPAS) was not administered for at least two days prior to the test.

A. Reproducibility of the microbiologic assay test for isoniazid. The reproducibility of the microbiologic assay test for isoniazid was investigated by performing the serial twofold tube dilutions containing known concentrations of isoniazid, inoculating the tubes with suspensions of dispersed, vigorously growing H37RvRSM organisms (RSM = resistant to 2.0 μg. streptomycin per ml. and susceptible to isoniazid), incubating at 36° C. for five days, and reading the results in terms of loss of acid-

TABLE I

Effect of various drugs on antimicrobially-active serum isoniazid level at six hours after a 4 to 8 mg. per Kg. test dose of isoniazid

Drug	Patients	Dosage, and time of administration related to test dose	Number of patients with: Increase	Number of patients with: No detectable change
1. PAS	140	2 Gm. at 0 and 3 hrs.	116	24
	5	1 Gm. at 0 and 3 hrs.	0	5
	5	5 Gm. at 0 hrs.	2	3
	5	2 Gm. at 0 hrs.	1	4
2. PABA	71	2.5 Gm. at 0 and 3 hrs.	65	6
3. Probenecid	5	2 Gm. daily for 2 days 500 mg. at 0 and 3 hrs.	0	5
4. Phenylbutazone	3	300 mg. at 0 and 3 hrs.	0	3
	2	600 mg. at 0 hrs.	0	2
5. Barbituric acid	3	2.5 Gm. at 0 and 3 hrs.	0	3
6. Phenobarbital	2	60 mg. 2 hrs. before 60 mg. at 0 and 3 hrs.	0	2
7. Secobarbital	2	45 mg. 2 hrs. before 45 mg. at 0 and 3 hrs.	0	2
8. Amobarbital	2	90 mg. 2 hrs. before 90 mg. at 0 and 3 hrs.	0	2
9. Glucosamine	3	3.5 Gm. at 0 and 3 hrs.	0	3
10. Chlorpromazine	6	25 mg. at 0 and 3 hrs.	0	6
11. Prednisone	5	25 mg. at 0 and 3 hrs.	0	5
12. Sodium salicylate	5	2.5 Gm. at 0 and 3 hrs.	0	5
13. Pyridoxine	7	100 mg. daily for 5 days 100 mg. at 0 and 3 hrs.	0	7

fastness (*2*). Fifty such tests were performed by one individual during a four month period.

It should be noted that the microbiologic assay for isoniazid, originally described by Mandel and associates (*2*), and employed here, differs significantly from other methods described in the literature (*3, 21, 22*). Not only is it more specific for isoniazid by virtue of the fact that the end-point is determined by loss of acid-fastness [1] rather than by gross evidence of inhibition of growth, but it also requires a shorter period of time for its performance, *i.e.*, five days versus the 10 days required for these other methods.

B. Serum isoniazid levels at six hours after a single loading dose. The initial microbiologic assay test was performed on 254 hospitalized adult tuberculous patients.

C. Reproducibility of serum level studies repeated on the same individuals. Isoniazid serum level studies were repeated at least once on 163 patients. The interval between the first and subsequent studies was from one day to six months. The greatest number of such studies repeated on any one individual was five.

D. Effect of increasing the loading dose of isoniazid from 4 to 8 mg. per Kg. The effect of increasing the loading dose of isoniazid from 4 to 8 mg. per Kg. was studied in 54 patients whose initial serum levels had been 0.2 μg. or more of isoniazid per ml. of serum at six hours following a 4 mg. per Kg. test dose.

E. Comparison of a "rapid" with a "slow" inactivator of isoniazid by both chemical and microbiologic methods. Serum levels of antimicrobially-active isoniazid as well as *isoniazid plus its derivatives* were determined on serial specimens of sera from two patients: One *achieved a low and the other a high* level following the 4 mg. per Kg. test dose. The method of determination of isoniazid plus its derivatives was based on assay for isonicotinic acid after complete hydrolysis and oxidation, and was reported as isoniazid equivalent (*10*). Blood was drawn for analysis at zero, two, four, six and 24 hours following an 8 mg. per Kg. test dose of isoniazid. The same study was repeated with 2.5 Gm. of para-aminosalicylic acid (2.85 Gm. NaPAS) added to the test dose of isoniazid. Para-aminosalicylic acid, 2.5 Gm., was also given at three, six and nine hours after the test dose.

F. Effect of various drugs on the serum isoniazid level. The effects of 13 different compounds on the antimicrobially-active serum isoniazid level were studied. Determinations were made on 160 serum specimens from 79 patients selected at random whose initial determinations revealed active serum levels below 0.4 μg. per ml. At the start of the drug testing program, the lowest dilution of serum employed was one to 10, but one to five dilutions were included later so that lower concentrations of isoniazid could be detected. All drugs except isoniazid were discontinued for at least one day prior to the test. On the test day, the patient usually was given a 4 mg. per Kg. dose of isoniazid plus one-half of the total dose

[1] Following the report that streptomycin administered first could prevent the isoniazid-produced loss of acid-fastness, this was studied in the sera from some of our patients. The presence or absence of streptomycin in the serum did not alter the result of the microbiologic assay for isoniazid, because a streptomycin-resistant strain was employed.

of the other drug at zero time. The second portion of the test drug was given three hours later (three hour time), and the blood was drawn for analysis three hours later (six hour time). This procedure varied slightly with different drugs and in different subjects. Control determinations without the administration of the test drug were usually performed within one week of the test. For comparative purposes, an attempt was made to use various drugs in the same subject when it was found that one drug increased the antimicrobially-active serum level of isoniazid. The drugs used, the dosages given, and the number of patients studied are listed in Tables I and II.

G. Comparison of isoniazid levels in serum and in cavity content. Surgical resections of closed cavities filled with caseous necrotic debris were performed on five patients. The specimens were removed approximately six hours after a 4 mg. per Kg. oral loading dose of isoniazid. At the same time blood was drawn so that a comparison could be made between the antimicrobially-active drug level in serum and in cavity content.

H. Microbiologic assay test for streptomycin. The concentration of streptomycin in sera was determined microbiologically by a filter-paper disc-seeded agar diffusion method similar to that described by Forgacs, Kornegay and Henley (23). Bacto antibiotic medium 5 (Difco Laboratories, Inc., Detroit, Mich.), pH 8, was used as the test medium, and *Bacillus subtilis* "I" strain (obtained from the Army Medical Department, Research and Graduate School, Walter Reed Hospital, Washington, D.C.) was the test organism. An important modification of previously described filter-paper disc agar diffusion methods was employed in this study: The final seeded plate with test discs in place was stored at 4° C. for 48 hours before incubation at 36° C. This greatly improved the sensitivity and accuracy of the method and permitted the use of undiluted sera for impregnation of the filter-paper discs. It permitted estimation of streptomycin concentrations between 5 and 50 μg. per ml. of serum. For each series of assays, a standard curve was obtained relating diameters of zones of growth inhibition to known concentrations of streptomycin, 5.6, 11.2, 22.5 and 40 μg. of streptomycin per ml. in duplicate.

I. Serum streptomycin levels at six hours after a single intramuscular loading dose. Each of 116 patients was given streptomycin sulfate, 20 mg. per Kg. of body weight, intramuscularly, and six hours later blood was drawn for testing. The venous blood sample was allowed to clot at room temperature, the clot was separated aseptically, and the serum stored at 4° C. until testing (within one week).

J. Reproducibility of serum streptomycin level studies on the same individuals. Repeat studies were performed on 157 patients under the same conditions at intervals of one day to seven months.

K. Correlation of in vitro drug resistance with serum streptomycin levels achieved by patients excreting resistant organisms. The precise level of streptomycin resistance manifested by tubercle bacilli recovered from the sputum of 33 patients who were excreting organisms resistant to streptomycin on admission was studied quantitatively. The populations of tubercle bacilli from 16 of these patients were completely resistant to 2.0 μg. of streptomycin per ml., but less than 50 per cent were resistant to 10 μg. of streptomycin per ml. It was considered possible that this low level of resistance might have resulted from failure of these 16 patients to achieve sufficiently high serum streptomycin levels during previous treatment with this drug. The streptomycin serum levels of these 33 patients at six hours after a 20 mg. per Kg. intramuscular loading dose of streptomycin were determined and correlated with the bacterial resistance findings.

TABLE II

The effect of para-aminosalicylic acid (PAS) and para-aminobenzoic acid (PABA) on the antimicrobially-active serum isoniazid level in nine patients

Patients	Time of determination after 4 mg. per Kg. test dose of isoniazid	Isoniazid alone	Isoniazid with PAS	Isoniazid with PABA
	hrs.	*μg./ml.*	*μg./ml.*	*μg./ml.*
L.S.*	3	0.8	2.4	3.2
	6	0.4	0.6	3.2
D.P.†	3	0.8	1.6	1.6
	6	0.4	0.4	0.8
F.H.†	3	0.8	0.8	0.8
	6	0.2	0.4	1.6
P.Y.†	3	0.6	0.8	2.4
	6	0.2	0.2	0.6
A.S.†	3	0.8	1.2	1.6
	6	0.2	0.4	1.6
M.S.*	6	0.4	0.4	1.2
E.C.*	6	0.4	0.8	1.6
O.R.†	6	0.4	1.2	1.6
A.P.†	6	0.2	0.2	0.4

* Five Gm. PAS or PABA given with test dose.
† Two and five-tenths Gm. of PAS of PABA given with test dose and three hours later.

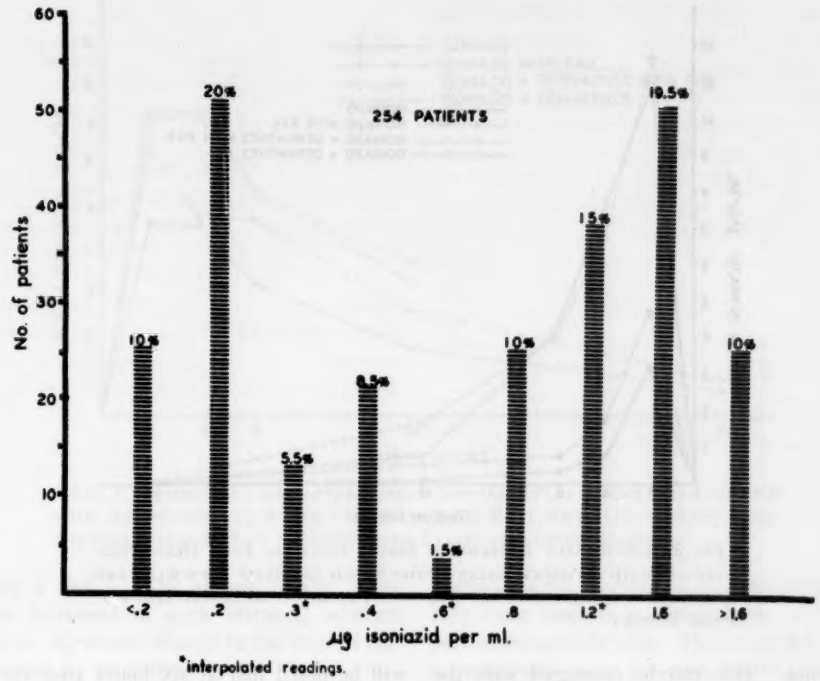

FIG. 1. SERUM ISONIAZID LEVELS
At six hours after 4 mg. isoniazid per Kg.

RESULTS

A. Reproducibility of the microbiologic assay test for isoniazid

Analysis of 50 consecutive microbiologic assay tests with the test strain of H37RvRSM employed throughout these studies, and with known concentrations of isoniazid, gave a mean value of 0.037 μg. isoniazid per ml. of test medium as the minimal concentration of isoniazid causing 50 per cent loss of acid-fastness under the conditions described. These results were obtained by the same individual over a period of four months. The standard deviation of the results of this series provides an estimation of the accuracy of the microbiologic assay method: ± 0.007 μg. isoniazid per ml. (± 20 per cent).

B. Serum isoniazid levels at six hours after a single loading dose

The distribution of values for antimicrobially-active levels of serum isoniazid at six hours after an oral test dose of 4 mg. isoniazid per Kg. of body weight in 254 hospitalized adult tuberculous patients is presented in Figure 1. The distribution curve of results has a clear-cut bimodal character: Approximately 55 per cent of these patients had levels above 0.6 μg. isoniazid per ml. of serum with a peak between 1.2 and 1.6 μg. per ml., and 45 per cent, lower than this value, with a peak near 0.2 μg. per ml. About 10 per cent of these patients were very rapid "inactivators" of the drug, having levels less than the minimum detectable level of 0.2 μg. per ml.

C. Reproducibility of serum level studies repeated on the same individuals

Isoniazid serum level studies at six hours after loading dosage of 4 mg. isoniazid per Kg. were repeated once on 163 patients after one day to six month intervals. The standard deviation from the mean of the isoniazid serum level results of the two studies on each patient was ± 28 per cent throughout the range 0.2 to 4.8 μg. isoniazid per

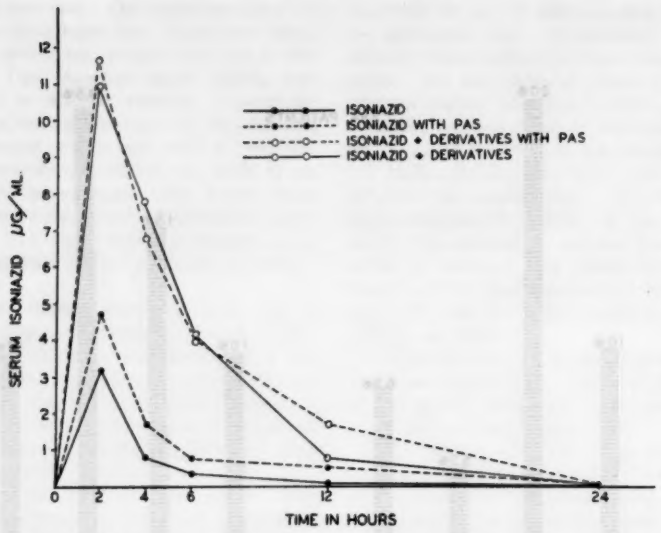

FIG. 2. CHEMICALLY DETERMINED SERUM ISONIAZID PLUS DERIVATIVES COMPARED WITH ANTIMICROBIALLY-ACTIVE SERUM ISONIAZID BOTH WITH AND WITHOUT PARA-AMINOSALICYLIC ACID IN A PATIENT WHO INACTIVATED ISONIAZID RAPIDLY

ml. of serum. This can be compared with the standard deviation from the mean which is inherent in the microbiologic testing procedure itself, ± 20 per cent.

D. Effect of increasing the loading dose of isoniazid from 4 to 8 mg. per Kg.

Isoniazid serum levels were obtained at six hours after a 4 mg. per Kg. loading dose and after an 8 mg. per Kg. loading dose in each of 54 patients. Only those individuals were included in this study whose serum levels after the 4 mg. per Kg. loading dose were 0.2 µg. or more isoniazid per ml. of serum. Fifty-one of these patients had serum level increases, and the average increase was 125 per cent, or somewhat more than doubling of the serum level after doubling the loading dose of drug in this dosage range. Only three patients had no demonstrable increase.

E. Comparison of a "rapid" with a "slow" inactivator

The results of the determinations of isoniazid plus all its derivatives in a "rapid" and a "slow" inactivator are presented in Figures 2 and 3. It will be noted that at six hours after the loading dose of isoniazid approximately 56 per cent of the total serum isoniazid was in an antimicrobially-active form in the "slow" inactivator, whereas only 5 per cent was in this form in the "rapid" inactivator. Over the 24 hour period, the "slow" inactivator had approximately five times more antimicrobially-active drug in the serum, expressed as µg. hours per ml. of serum, than did the "rapid" inactivator.

F. Effect of various drugs on the isoniazid level

The effect of the drugs tested is presented in Tables I, II and III. The original observation on the effect of administration of para-aminosalicylic acid (PAS) on the metabolism of isoniazid in human subjects was reported elsewhere by us (2). This observation has been confirmed by Morse and co-workers (3) and by Bell and Mitchell (22). Dosages of para-aminosalicylic acid of 2.5 Gm. given at the zero and three hour times were more effective than the other dosage schedules studied.

The effects of para-aminosalicylic acid on the antimicrobially-active serum isoniazid level in a "rapid" and a "slow" inactivator have been shown

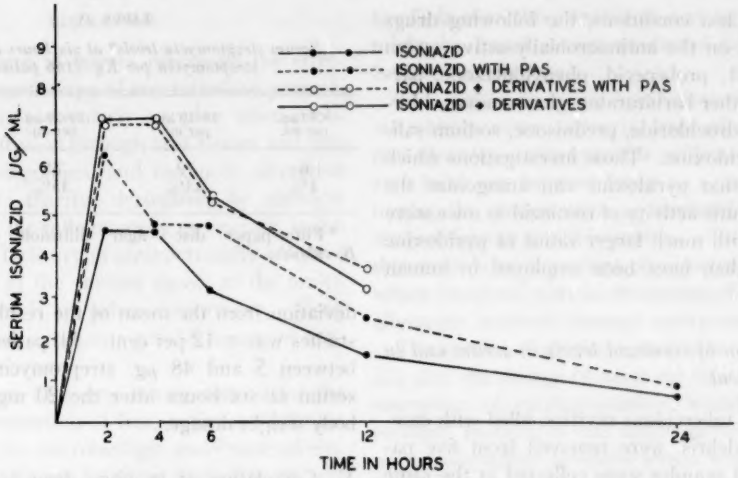

FIG. 3. CHEMICALLY DETERMINED SERUM ISONIAZID PLUS DERIVATIVES COMPARED WITH ANTIMICROBIALLY-ACTIVE SERUM ISONIAZID BOTH WITH AND WITHOUT PARA-AMINOSALICYLIC ACID IN A PATIENT WHO INACTIVATED ISONIAZID SLOWLY

in Figures 2 and 3. The antimicrobially active level was increased in each instance, whereas there was no significant change in the level of isoniazid plus all its derivatives.

It has been demonstrated that: *1*) The addition of para-aminosalicylic acid (1,000 µg. per ml.) to the microbiologic assay medium does not modify the antimicrobial activity of isoniazid; *2*) The addition of para-aminosalicylic acid to serum from patients receiving isoniazid does not alter the results of this test for antimicrobially-active isoniazid; and *3*) The antimicrobial activity of isoniazid is not modified *in vitro* by serum (Table III) from patients receiving large oral doses of para-aminosalicylic acid. Therefore, the method of microbiologic assay prevents the *in vitro* antimicrobial action of para-aminosalicylic acid.

It is clear (Tables I and II) that the addition of para-aminobenzoic acid which has no direct antimycobacterial action led to a detectable increase in the antimicrobially-active serum isoniazid level in the 71 subjects tested, and that this increase was generally higher and more sustained than that achieved by para-aminosalicylic acid.

TABLE III

Effect of orally administered para-aminosalicylic acid (PAS) on results of microbiologic assay for isoniazid (INH) in serum

Subject	INH administered orally 18 hours previously				INH 8 mg./Kg. administered orally 6 hours previously	
	No addition of INH to serum		INH, 0.4 µg. added per ml. of serum			
	Without oral PAS	With oral PAS*	Without oral PAS	With oral PAS*	Without oral PAS	With oral PAS, 2.5 Gm. at 0 and 3 hrs.
A.L.	<0.2†	<0.2	0.4	0.6	0.4	1.2
O.D.	0.2	0.2	0.6	0.8	0.8	3.2
O.R.	0.2	0.2	0.8	0.8	0.8	2.4
E.G.	0.4	0.4	0.8	0.8	4.8	4.8
S.K.	0.4	0.4	0.8	0.8	3.2	6.4
W.B.	0.4	0.4	0.8	0.8	1.6	3.2
W.R.	<0.2	<0.2‡	0.2	0.4‡	2.4	4.8

* PAS, 10 Gm. orally in one dose; blood collected at two and one-half hours.

† Micrograms INH per ml. of serum specimen, by microbiologic assay.

‡ Special dosage; PAS, 24 Gm. (30 Gm. NaPAS), taken 12 Gm. at zero hour and 12 Gm. at three hours; blood collected at six hours.

Under the test conditions, the following drugs had no effect on the antimicrobially-active serum isoniazid level: probenecid, phenylbutazone, barbituric acid, other barbiturates, glucosamine, chlorpromazine hydrochloride, prednisone, sodium salicylate and pyridoxine. Those investigations which have shown that pyridoxine can antagonize the chemotherapeutic activity of isoniazid in mice were carried out with much larger ratios of pyridoxine to isoniazid than have been employed in human beings (21).

G. Comparison of isoniazid levels in serum and in cavity content

Obstructed tuberculous cavities, filled with caseous necrotic debris, were removed from five patients. Blood samples were collected at the same time. In each of these patients the serum level of antimicrobially-active isoniazid at six hours was the same, within the limits of error, as the level in the cavity content.

H. Reproducibility of the microbiologic assay test for streptomycin

Analysis of the duplicate results of 23 consecutive microbiologic assays for known concentrations of streptomycin sulfate gave the following results: mean, 5.6 μg. per ml. (standard deviation, ± 6 per cent); mean, 11.2 μg. per ml. (standard deviation, ± 7 per cent); mean, 22.5 μg. per ml. (standard deviation, ± 8.5 per cent); mean, 45 μg. per ml. (standard deviation, ± 11 per cent). These results were obtained by the same individual over a period of seven months.

I. Serum streptomycin levels at six hours after a single intramuscular loading dose

The distribution of values for antimicrobially-active levels of serum streptomycin at six hours after an intramuscular test dose of 20 mg. streptomycin sulfate per Kg. of body weight in 166 hospitalized adult tuberculous patients are presented in Table IV.

TABLE IV

Serum streptomycin levels at six hours after 20 mg. streptomycin per Kg. (166 patients)*

<10 μg. per ml.	10–19 μg. per ml.	20–29 μg. per ml.	30 or >μg. per ml.
6 4%	72 43%	58 35%	30 18%

* Filter-paper disc — agar diffusion method using *B. subtilis*.

J. Reproducibility of serum streptomycin level studies on the same individuals

Among the 157 patients who received loading dosage of 20 mg. streptomycin per Kg. of body weight on two different occasions, the standard deviation from the mean of the results of the two studies was ± 12 per cent. All patients had levels between 5 and 48 μg. streptomycin per ml. of serum at six hours after the 20 mg. per Kg. of body weight dosage.

K. Correlation of in vitro *drug resistance with serum streptomycin levels achieved by patients excreting resistant organisms*

The results of this study are presented in Table V. Statistical analysis of the difference between the two groups shows that it is highly significant ($p < 0.01$).

TABLE V

Correlation of streptomycin serum levels and levels of streptomycin resistance of tubercle bacilli recovered from 33 tuberculosis patients previously treated with streptomycin

Group 1*		Group 2†	
Patient	Streptomycin serum level‡	Patient	Streptomycin serum level‡
	μg./ml.		μg./ml.
L.A.	5	B.S.	15
R.W.	5	F.B.	20
L.W.	9	R.E.	20
M.B.	10	D.G.	20
K.C.	10	E.M.	20
I.G.	10	F.N.	20
B.L.	10	O.R.	20
L.S.	10	G.S.	20
W.A.	15	G.B.	30
R.N.B.	15	G.O.	30
M.M.	15	G.S.	30
H.S.	15	H.W.	30
R.B.	20	M.S.	35
W.R.	20	G.C.	40
G.W.	20	J.H.	40
C.B.	30	G.M.	40
		P.H.	50

* Excreting populations resistant to 2 μg. streptomycin per ml. and less than 50 per cent resistant to 10 μg. streptomycin per ml.

† Excreting populations resistant to 2 μg. streptomycin per ml. and more than 50 per cent resistant to 10 μg. streptomycin per ml.

‡ At six hours after a loading does of 20 mg. streptomycin sulfate per Kg. of body weight.

DISCUSSION

A wide variety of factors determine the effectiveness of chemotherapy of any infectious process. The situation is particularly complex when problems such as diffusion through host tissues and into phagocytes, antagonism, and metabolic alteration of the agent to inactive derivatives by chemical components of the inflammatory or necrotic lesion are involved. Delivery of antimicrobially-effective concentrations of the various agents to the multiplying parasites is of prime importance. Drug serum levels may serve as a reliable means of estimating such delivery.

In the absence of a satisfactory method for the chemical determination of free, antimicrobially-active isoniazid, the microbiologic assay method employed here is shown by these studies to be sufficiently accurate, simple and reproducible for clinical purposes. The wide variations in the rate and degree of biochemical alteration of the isoniazid molecule in different subjects, and the relative constancy for a given individual, as previously reported (3, 9, 22), have been confirmed. Also, there is no doubt that higher dosages of isoniazid lead to higher serum levels of antimicrobially-active drug.

Certain clinical observations (24) suggested that daily dosages of isoniazid higher than 3 to 5 mg. per Kg. of body weight should result in better chemotherapeutic response. However, there was no experimental evidence of a relationship between serum levels of this drug and response of human beings to treatment until our demonstration (9) of a highly significant relationship between serum isoniazid levels and the catalase activities of drug-resistant tubercle bacilli from isoniazid-treated patients. Then, it became evident that the serum level of antimicrobially-active isoniazid achieved in an individual depends on whether the subject is a "slow" or "rapid" inactivator as well as on the dosage of drug that he receives.

Approximately 45 per cent of the 254 subjects tested failed to achieve a level of at least 0.8 μg. per ml. at six hours after a 4 mg. per Kg. dose. Inasmuch as a high proportion of the patients studied were known drug treatment failures on admission to National Jewish Hospital, and failure can be a consequence of inadequate delivery of at least one of the drugs to the multiplying tubercle bacilli *in vivo*, this percentage may be higher than might be found in an unselected population of tuberculous patients.

The studies described here comparing the antimicrobially-active isoniazid level of serum with that of cavity content provide evidence that the serum level can be used fairly as a basis for estimating the concentration of isoniazid delivered to the multiplying bacilli in cavities. They also show that there is no important physicochemical barrier which interferes with the movement of antimicrobially-active isoniazid through cavity walls.

The results of previous studies have shown (9, 25) that the dosage of isoniazid which avoids the emergence of catalase-positive, weakly isoniazid-resistant mutants is one which provides an antimicrobially-active isoniazid serum level of at least 0.4 μg. per ml. at six hours following one-half of the total daily dose. As previously indicated (1), this dosage yields drug levels *in vivo* which are much higher than necessary to avoid the emergence of any drug-resistant mutants when a second drug is employed, such as streptomycin, also in adequate dosage on combined drug treatment. Therefore, an optimal chemotherapeutic response may be anticipated (and with a wide margin of safety) in original treatment patients excreting drug-susceptible populations of tubercle bacilli if one-half of the total daily dose of isoniazid gives a serum level of at least 0.4 μg. per ml.

An increase in the level of antimicrobially-active drug to insure that all patients will maintain adequate serum levels of isoniazid may be accomplished either by increasing the dosage of isoniazid or by employing drugs which apparently prevent rapid inactivation of the drug. An increase in dosage of isoniazid to 16 to 24 mg. per Kg. daily without pyridoxine has led to a prohibitive increase in isoniazid neuropathy (24). The simultaneous administration of pyridoxine with isoniazid has reduced but not completely prevented toxicity due to isoniazid (24, 26). Some few subjects inactivate isoniazid so rapidly that dosages of 16 to 20 mg. per Kg. per day are insufficient to maintain adequate, antimicrobially-active, serum isoniazid levels. It is in these patients that high dosage plus the concomitant administration of drugs such as para-aminosalicylic acid or para-aminobenzoic acid may be employed. A decrease in the rate of metabolic alteration of isoniazid by these drugs has been attributed to competition for the biochemical proc-

ess of acetylation (27, 28). The action of para-aminosalicylic acid on the antimicrobially-active serum isoniazid level suggests that the effectiveness of combined therapy employing isoniazid and para-aminosalicylic acid (29) is due not only to the antimicrobial activity of each drug, but also to the resulting elevation of the antimicrobially-active serum isoniazid level.

It seems fair to conclude that to achieve optimal therapeutic results and to reduce the incidence and severity of the toxic effects of the drug, serum levels of antimicrobially-active isoniazid should be known. The dosage of isoniazid which should be employed is clearly related to the rate at which the patient inactivates the drug; and it would seem that this can be measured satisfactorily by the microbiologic assay method.

While streptomycin does not appear to be subject to metabolic alteration in man (15), the problem of adequate delivery of the drug to multiplying tubercle bacilli is very complicated (30). Not all intramuscular injections (as routinely administered) are actually into muscle tissue. Absorption from such an "intramuscular" depot is influenced by factors such as inflammatory response and blood supply. Some subjects excrete streptomycin more rapidly than others. All other factors being equal (which they seldom are), heavy subjects should achieve relatively lower tissue concentrations than light subjects on a conventional 1.0 Gm. dosage schedule. There are also chemical components of inflammatory or necrotic tissue, such as nucleic acids, which antagonize the antimicrobial action of streptomycin (31–33). Finally, the activity of streptomycin is modified by the concentration of viable tubercle bacilli exposed to it. Such uncontrollable variables render hazardous any attempt to correlate the concentration of streptomycin achieved in the serum with the actual delivery of the drug in an active form to tubercle bacilli in patients.

In spite of the uncontrollable modifying factors, it was considered important to know that at least the first step in the delivery of sufficient concentrations of streptomycin had been achieved. Accordingly, serum levels of antimicrobially-active streptomycin sulfate were determined. These confirmed previous reports that variations do exist between subjects (16, 34).

The results of the studies which relate the levels of drug resistance of streptomycin-resistant mutants excreted by patients previously treated with streptomycin and the concentrations of streptomycin which are found in their sera after the standard test are significant. As in previous similar studies with isoniazid (9), these observations establish the six hour serum assay for streptomycin as a reliable basis for estimating the concentration of this drug delivered to multiplying tubercle bacilli in most patients. Thus, in spite of the indeterminable local factors which influence the antimicrobial activity of streptomycin, humoral levels of the drug play an important role.

SUMMARY

The results of microbiologic assays for antimicrobially-active isoniazid in serum and tuberculous cavity content are presented. Some effects of change in dosage of isoniazid and of concomitant administration of various other drugs on the antimicrobially-active serum isoniazid levels in patients are reported. Para-aminosalicylic acid and para-aminobenzoic acid were the only drugs studied that significantly increased this level.

The results of application of a microbiologic assay method for serum streptomycin are also presented, and, as previously shown for isoniazid, a significant relationship is demonstrated between serum levels of streptomycin and certain aspects of the microbiologic response in patients to streptomycin treatment.

The clinical implications of the variations in antimicrobially-active serum levels of both isoniazid and streptomycin achieved by different patients are discussed.

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COMBINED DRUG TREATMENT OF TUBERCULOSIS. III. CLINICAL APPLICATION OF THE PRINCIPLES OF APPROPRIATE AND ADEQUATE CHEMOTHERAPY TO THE TREATMENT OF PULMONARY TUBERCULOSIS

By WILLIAM F. RUSSELL, Jr., IRVING KASS, ANGELINE D. HEATON, SIDNEY H. DRESSLER and GARDNER MIDDLEBROOK

(From the Department of Medicine and Research and Laboratories, National Jewish Hospital at Denver, and the Departments of Medicine and Microbiology, University of Colorado School of Medicine, Denver, Colo.)

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Microbiologic investigation of the prevention of emergence of mutant populations of tubercle bacilli resistant to streptomycin and isoniazid emphasized the fact that adequate concentrations of each of these two appropriate drugs were necessary (1). Delivery of such concentrations of isoniazid to the multiplying parasites can be estimated by microbiologic assay (2–4). Antimicrobially-active serum streptomycin assayed by a similar microbiologic method confirmed that there were also probably significant variations between individuals in serum levels of this drug as well. These observations suggested that conventional dosages of these drugs could not be relied upon to insure the delivery *in vivo* of sterilizing concentrations of each agent to physiologically-susceptible tubercle bacilli. The result of consideration of these principles was the formulation of a chemotherapeutic regimen combining high dosage of streptomycin with high dosage of isoniazid for all patients (5–7). The purpose of this report (the third of a series) is to present the results of clinical application of this approach to chemotherapy of pulmonary tuberculosis.

METHODS AND MATERIALS

A. Bacteriology. The bacteriologic studies were performed according to methods described elsewhere (8, 9). These routine studies consisted of Ziehl-Neelsen stained smears of the concentrated, digested sputum; the results were reported as the number of acid-fast rods seen per oil immersion field. The concentrated sediment of the digested sputum was then diluted according to the number of acid-fast rods seen on smear. When the smear revealed less than one acid-fast rod per oil immersion field, the concentrate was inoculated both undiluted and diluted 10^{-1}. When the concentrate smear revealed one to 10 acid-fast rods per oil immersion field, the sediment was diluted 10^{-1} and 10^{-2}. When the smear revealed more than 10 acid-fast rods per oil immersion field, the sediment was diluted 10^{-2} and 10^{-3} prior to inoculation of the culture plates. Every specimen was seeded on solid medium (1) containing no antimycobacterial drugs (the control), on the same medium containing streptomycin, 2.0 μg. per ml., and on the same medium containing isoniazid, 0.2 μg. per ml. During the period covered in this study, a third direct routine drug-susceptibility test was performed. Prior to 1955, this third test was isoniazid, 10 μg. per ml. In 1955, pyrazinamide, 50 μg. per ml., was the test drug for some months. Since that time, para-aminosalicylic acid, 2.0 μg. per ml., has been used in this third susceptibility test.

The definition of drug-susceptibility employed in this report is as follows: A strain or population of tubercle bacilli is susceptible to the action of streptomycin or isoniazid if less than 1 per cent of those members yielding colonies on the control are able to grow and produce colonies on culture medium containing either streptomycin, 2.0 μg. per ml. or isoniazid, 0.2 μg. per ml. Such bacterial populations were considered significantly drug-resistant if more than 1 per cent was recovered on medium containing streptomycin or isoniazid in these concentrations.

Catalase testing, using 15 per cent hydrogen peroxide in 5 per cent Tween® 80, was performed routinely on the control culture (8, 9). As employed, this test provides a crude estimate of the moderate catalase activity of typical tubercle bacilli, the high activity of the so-called "yellow" bacilli, and the reduced or absent activity of catalase-deficient, isoniazid-resistant mutants.

The culture plates were incubated under 2 to 5 per cent carbon dioxide in air at 36° C. for 21 days (10). The results of culture, drug-susceptibility testing, and catalase testing became available for clinical use within 25 days following collection of the sputum. This provided a unique opportunity for the selection of the appropriate agents and for close monitoring of the bacteriologic response of the patient.

Bacteriologic conversion of the sputum to negative was defined to have occurred when three consecutive sputum specimens collected at monthly intervals were found to be negative both on smear of the concentrate and on culture, and no subsequent positive sputum studies were encountered. The date of conversion was that of the first specimen which was negative on both smear and culture. This

TABLE I

Cumulative bacteriologic conversion (chemotherapy alone)

Group	Total	Negative at 1–4 mos./total	Negative at 5–8 mos./total	Negative at 9–12 mos./total
I	(153)	127/153	152/153	141/142*
II	(39)*	12/39*	15/36*	16/34*
III	(117)*	11/117*	13/105*	14/82*

* Losses due to early discharge or conversion concomitant with surgery or collapse procedures.

has been reported as occurring in the one to four month period (from Day 1 through Day 120 of chemotherapy), the five to eight month period (from Day 121 through Day 240 of chemotherapy), the nine to 12 month period, and so forth. When sputum conversion occurred as the result of collapse procedures (pneumothorax, pneumoperitoneum or thoracoplasty) or resection surgery, the subject was eliminated from further bacteriologic statistical analysis; this accounts for some of the reduction in actual cases observed in Table I.

B. Roentgenography. Admission roentgenograms of the chest were interpreted according to the Diagnostic Standards of the National Tuberculosis Association, 1955 (11), with the following differences: *1)* Cavities have been reported as single or multiple, and *2)* cavity size has been reported as the greatest internal dimension of the largest single cavity. Thus, in some instances, *moderately advanced* disease has been reported here associated with a large cavity or with multiple small cavities the sum of the diameters of which would require designation as *far advanced* by N.T.A. Standards. This modification was necessary because it seemed important to analyze the extent of disease separate from the cavity component.

X-rays made during the one to four month period and the five to eight month period have been compared with the admission film in order to assess the response to treatment. Comparison roentgenograms were selected which were made near to the end of the period. However, when collapse procedures or resection surgery were employed, the film taken just prior to the procedure was used for the final comparison and no further X-ray evaluation was made for these studies. This accounts for some of the reduction in total cases observed in Tables II and III.

Special roentgenographic studies, including tomography, were performed early during the admission of each patient, and were repeated as indicated.

C. Microbiologic assay for serum isoniazid. Estimation of the serum concentration of antimicrobially-active isoniazid has been an important part of the evaluation of our patients during most of the period covered in this study. Determinations were made by the microbiologic method in 280 of the patients being reported here (12, 13). These patients were divided into three categories on the basis of their serum levels of active isoniazid. Thus, 76 had levels less than 0.4 μg. per ml.; 94 had levels of 0.4 to 0.8 μg. per ml.; and 110 had levels in excess of 0.8 μg. per ml. It has been estimated that a serum level of active isoniazid of 0.4 μg. or more per ml. at six hours after one-half of the daily dose is adequate for optimal chemotherapeutic action (2).

When it was shown that concomitant administration of

TABLE II

General roentgenographic response to chemotherapy

Group	Total		Worse		None–slight improvement		Mod.–marked improvement	
	1–4 mos.	5–8 mos.	1–4 mos.	5–8 mos.	1–4 mos.	5–8 mos.	1–4 mos.	5–8 mos.
I	153	153	1	1	83	49	69	103
II	40	37*	0	2	34	23	6	12
III	123	99*	3	5	116	87	4	7

* Losses due to collapse procedures and/or resection surgery.

TABLE III

Roentgenographic response of cavities to chemotherapy

Group	Total		Cavity status							
			Present							
			Open		Filled		Indefinite		Lost to view	
	1–4	5–8	1–4	5–8	1–4	5–8	1–4	5–8	1–4	5–8
I	145	145	117	71	3	12	2	11	23	51
II	40	38*	37	30	0	3	2	2	1	3
III	118*	94*	112	79	3	9	1	1	2	5

* Losses due to collapse procedures and/or resection surgery.

TABLE IV

*Criteria for selection of patients**

Total adult pulmonary tuberculosis cases		606
Eliminated for following reasons:		−288
1. Sputum negative, consistently	184	
2. Sputum positive (minimally), with unsatisfactory drug-susceptibility studies	41	
3. Sputum positive, "atypical yellow bacilli"	16	
4. Sputum positive, typical tubercle bacilli, treated less than 6 months—Dead	8†	
—Irregular discharge	39	
	288	
All bacteriologically proven pulmonary tuberculosis patients excreting typical tubercle bacilli, treated at least 6 months		318

* October 1, 1954, to June 1, 1957.
† Five nontuberculous deaths; three surgical deaths, all excreting drug-resistant organisms.

para-aminosalicylic acid (or para-aminobenzoic acid) elevated the serum levels of active isoniazid, this drug (in the dosage of 12 Gm. NaPAS or NaPABA daily) was added to the basic regimen of those patients known to have levels less than 0.4 μg. per ml. (12, 13). The specific purpose of adding either of these drugs to the basic regimen in these individuals was to elevate the serum level of active isoniazid.

D. Microbiologic assay for serum streptomycin. Serum levels of antimicrobially-active streptomycin have been determined in 48 of these patients. There were four who had levels less than 10 μg. per ml.; 13 had levels of 10 μg. per ml.; 23 had levels of 20 μg. per ml.; and eight achieved levels in excess of 20 μg. per ml. The microbiologic method used was a serial tube dilution technique employing an isoniazid-resistant strain of H37Rv. This method has yielded generally lower values than the filter-paper disc—agar diffusion method using a strain of *B. subtilis* (3). Limited correlation of chemotherapeutic response with streptomycin levels at six hours following a 20 mg. per Kg. test dose suggested that patients who fail to achieve a level in excess of 20 μg. per ml. were receiving what might prove to be insufficient dosage. Thus, in this study, several patients in Group I (see below) who had "adequate" isoniazid serum levels and had not converted to negative before 10 weeks after initiation of chemotherapy and who had streptomycin serum levels below 20 μg. per ml. were given increased dosage of streptomycin up to 35 mg. per Kg. per day.

E. Clinical material. Between October 1, 1954, and June 1, 1957, a total of 606 patients with the clinical diagnosis of pulmonary tuberculosis were admitted consecutively to the adult service of National Jewish Hospital at Denver (Table IV). Of these, 288 were considered unsuitable for bacteriologic evaluation of the high dosage regimen. Sputa from 184 of these 288 patients were consistently negative, and sputa from 41 were so infrequently and so slightly positive that drug-susceptibility tests were unsatisfactory.[1] Sixteen patients were excreting "atypical yellow bacilli," *Mycobacterium tuberculosis luciflavum* (14). Of those patients excreting typical tubercle bacilli, eight were lost because of death (five nontuberculous deaths, and three surgical deaths; these latter were excreting drug-resistant tubercle bacilli), and 39 because of irregular discharge, after less than six months of treatment. This study is concerned with the results of high dosage chemotherapy of 318 adults admitted consecutively with bacteriologically-proven pulmonary tuberculosis, with typical tubercle bacilli recovered on sputum culture, and with treatment for at least six months.

On the basis of the results of drug-susceptibility testing of the populations of tubercle bacilli recovered from the admission sputa, these 318 patients have been divided

[1] One of these patients became consistently sputum-positive with streptomycin-resistant, isoniazid-resistant, para-aminosalicylic acid-susceptible, catalase-negative tubercle bacilli in the sputum four months after admission and while on chemotherapy with isoniazid/para-aminosalicylic acid.

TABLE V

Background data on 318 patients grouped according to drug-susceptibility studies

Group	Drug susc.	Sex	Number	Average age (yrs.)	Range	White	Nonwhite
I	SM–S/INH–S*	M	94	40.5	(15–68)	80	14
		F	59	37.6	(17–80)	50	9
II	SM–R/INH–S	M	26	41.5	(21–70)	24	2
	SM–S/INH–R†	F	14	28.8	(17–56)	14	0
III	SM–R/INH–R	M	71	44.6	(20–68)	67	4
		F	54	38.6	(20–69)	47	7

* –S, susceptible to streptomycin (SM), 2 μg. per ml., or isoniazid (INH), 0.2 μg. per ml.
† –R, resistant to SM, 2 μg. per ml or INH, 0.2 μg. per ml.

TABLE VI

Comparative roentgenographic characteristics

Extent of disease		Cavity status	Size	I	II	III
Minimal				1		2
Moderately advanced,	Uni,	No cavity		3		
		Sing.	0.5	4	2	2
			1–2	3	2	4
			2.5–3.5	1	1	5
			4.0–7.5	1		
		Mult.	1–2	6	1	2
			2.5–3.5	1		
	Bil,	No cavity		3		2
		Sing.	0.5	2		
			1–2	17	4	10
			2.5–3.5	10		7
			4.0–7.5	1	1	4
		Mult.	0.5	1		
			1–2	12	2	2
			2.5–3.5	17	1	8
			4.0–7.5	5		3
			8.0 or >	1		1
Far advanced,	Uni,	Sing.	4.0–7.5	1		
		Mult.	1–2			1
	Bil,	No cavity		1		1
		Sing.	1–2	4	6	6
			2.5–3.5	4	2	4
			4.0–7.5	4	1	6
			8.0 or >		1	
		Mult.	1–2	8	2	10
			2.5–3.5	14	5	13
			4.0–7.5	20	5	19
			8.0 or >	7	4	13
			Total	153	40	125

into three categories (Table V). Group I patients were excreting streptomycin-susceptible, isoniazid-susceptible populations of tubercle bacilli. Group II patients were excreting either streptomycin-resistant, isoniazid-susceptible or streptomycin-susceptible, isoniazid-resistant populations. Group III patients were excreting streptomycin- and isoniazid-resistant populations. All patients of Groups II and III (except possibly one) who were excreting isoniazid-resistant populations of tubercle bacilli gave histories of at least three weeks' previous treatment with isoniazid or its chemical derivatives. Similarly, all, except four patients, who were excreting streptomycin-resistant populations gave histories of previous streptomycin or dihydrostreptomycin treatment.

These three groups were similar with respect to age, sex, and race (Table V). Comparison of the members of these three groups of patients by roentgenographic characteristics of disease revealed that they were sufficiently comparable for the purposes of this study (Table VI).

F. Basic chemotherapeutic regimen. This consisted of streptomycin sulfate (Squibb), 1 to 2 Gm. daily (20 mg. per Kg. per day or more in those patients who had low streptomycin serum levels), by the intramuscular route in a single dose, for at least 90 days. Streptomycin was not discontinued until the sputum was negative on smear and culture of the concentrate. Isoniazid (Nydrazid®, Squibb), 16 or more mg. per Kg. per day, was administered orally for at least 18 months. Isoniazid was given in two equal doses each day. Pyridoxine hydrochloride (Squibb), 50 to 100 mg. per day, was also given orally for 18 months. Pyridoxine was included in this regimen to reduce the incidence of significant isoniazid neuropathy (15). It should be emphasized that following termination of streptomycin, the isoniazid and pyridoxine were continued alone.

During the period covered in this report, the effect of short-term, 21 day chemotherapy with pyrazinamide (2.5 Gm. per day) and cortisone (100 mg. per day), to be followed at once by the basic regimen, was under investigation in 37 of these patients. So far no significant difference in results of this strictly randomized study has been observed.

Treatment with the basic high dosage regimen was

TABLE VII

Basic chemotherapeutic regimen adaptations

Group	Drug-susc.	Chemotherapy
I	SM–S/INH–S	Basic high dosage regimen (SM/INH)*
II	SM–R/INH–S	INH/PAS
	SM–S/INH–R	SM/PAS plus INH
III	SM–R/INH–R	VM, PZA, PAS, TTC, SM, and INH*

* Para-aminosalicylic acid (PAS) or para-aminobenzoic acid (PABA) added if serum INH level < 0.4 μg. per ml.

TABLE VIII

Significant drug reactions

Drug	Manifestation	Number
Streptomycin (SM)	Dermatologic reactions*	4†
	VIIIth cranial nerve, vestibular dysfunction*	3
	Optic neuritis (?)	1
	Thrombocytopenic purpura, aggravated by INH	1
Isoniazid (INH)	Psychosis	4
	Neuropathy, incipient	12
	Dupuytren's contracture	2†
	Emotional storms	12
	Total	39 (12%)

* Severe reactions.
† Chemotherapy continued with steroids.

considered to be appropriate for the Group I patients (Table VII). Half of Group II patients were excreting streptomycin-resistant, isoniazid-susceptible organisms and isoniazid plus para-aminosalicyclic acid (NaPAS, 12 Gm. per day) was appropriate. The other half of Group II were excreting streptomycin-susceptible, isoniazid-resistant tubercle bacilli and daily streptomycin, 1 to 2 Gm. for at least 90 days with para-aminosalicylic acid (NaPAS, 12 Gm. per day) plus isoniazid, was deemed appropriate chemotherapy.

Patients in Group III were treated with viomycin, pyrazinamide, para-aminosalicylic acid, tetracycline and streptomycin in a variety of combinations with isoniazid. Most patients in Groups I and III who had active isoniazid levels less than 0.4 μg. per ml. were also given para-aminosalicylic acid or para-aminobenzoic acid to raise the isoniazid level. Isoniazid was included in every regimen because it was considered appropriate to prevent back-mutation. Both animal experimentation (16) and *in vitro* studies (17) have proven that back-mutation to isoniazid-susceptibility can occur.

After initiation of chemotherapy, all patients, except those with severely restricted cardiopulmonary function, were out of bed for 10 to 12 hours daily.

Collapse procedures and/or resection surgery were performed on Group II and III patients when deemed appropriate, but not on Group I patients until four months after conversion.

G. Drug reactions. Serious drug reactions with the high dosage regimen incorporating pyridoxine have been infrequent (Table VIII). Significant reactions to streptomycin or isoniazid occurred in 12 per cent of our patients. The specific drug was permanently discontinued only for those few patients manifesting severe vestibular nerve reactions, optic neuritis, aggravation of thrombocytopenic purpura or psychosis. No apparent change in clinical response to chemotherapy was observed in these patients following termination of the offending drug.

RESULTS

There was a striking difference in roentgenographic inprovement estimated separately from changes in cavity status between Group I and Groups II and III after four months of chemotherapy, and this became more marked during the five to eight month period (Table II). Two of the patients in Group III were subjected to thoracoplasty within 60 days of the start of chemotherapy, and so do not appear in this analysis. Similarly, three Group II patients and 24 Group III patients were eliminated from X-ray evaluation by the application of collapse procedures or surgical resection during the five to eight month period.

In 51 of the Group I patients with demonstrable cavitation on admission, no cavities could be detected on routine X-rays and tomograms by the end of five to eight months of chemotherapy (Table III). As a further evaluation, analysis of the changes in cavity status confirmed the superior response to chemotherapy of Group I.

One hundred and fifty-two Group I patients had sputum conversion to negative by the end of the sixth month of chemotherapy (Table I). No drug-resistant cultures were obtained from these patients prior to conversion. However, there was one chemotherapeutic failure, a 48 year old white man with far-advanced, bilateral cavitary disease. He had been treated with isoniazid, 300 mg. per day, and para-aminosalicylic acid, 12 Gm. per day, three days before admission to this hospital. The tubercle bacilli proved to be susceptible to streptomycin and isoniazid. His serum isoniazid level at six hours following a 4 mg. per Kg. test dose was 1.2 μg. per ml. His serum streptomycin level at six hours after a 20 mg. per Kg. test dose was 20 μg. per ml. Both levels can be considered adequate for optimal antimicrobial effect. His sputum became negative on smear and culture in less than 12 weeks of chemotherapy with isoniazid, 16 mg. per Kg. per day, and streptomycin, 20 mg. per Kg. per day. However, six weeks later his sputum suddenly reverted to positive with tubercle bacilli *simultaneously resistant* to both streptomycin and isoniazid; his sputum cultures remained positive.[2]

[2] The possibility that this was the result of exogenous reinfection was considered. Survey of all hospitalized patients excreting organisms resistant to streptomycin (SM) and isoniazid revealed only one likely candidate. However, the organisms from that patient were highly resistant to streptomycin, 16 μg. per ml., while the or-

TABLE IX

Cumulative bacteriologic conversion (collapse procedures and resection in addition to chemotherapy)

Group	Total	1–4 Mos.		5–8 Mos.		9–12 Mos.
I	(153)	127	(153)	152	(142)	141
II	(40)	13	(40)	19	(39)*	21
III	(125)	19	(125)	33	(107)*	39

* None of Group I was subjected to collapse or resection until at least four months after conversion. Losses shown are due to early discharge.

These results with chemotherapy alone for Group I patients are strikingly different from those of both Groups II and III. If the Group II patients whose sputum became converted to negative concomitantly with collapse procedures or resection surgery were considered together with those achieving the converted state by chemotherapy alone, the total conversions by the end of one year would be only slightly more than half of the cases (Table IX). Similarly, only about one-third of the Group III patients became converted to the sputum negative state by the end of one year of treatment.

Follow-up observations on Group I patients

Follow-up observations have been completed on 90 per cent of the first 113 Group I patients who were treated for at least six months. At the time of this study, these patients had been observed for an average of two years since the start of chemotherapy, ranging from 18 to 33 months. Fifty-three of these patients were still on chemotherapy at the time of follow-up study, and 51 had terminated all antimicrobial drugs. The average period of time between termination of chemotherapy and the mailing of the most recent follow-up questionnaire was five and four-tenths months, ranging from one to 15 months. Of those patients whose chemotherapy was discontinued, only one patient has had a relapse, both bacteriologic and roentgenographic. This 39 year old white man had partial gastrectomy, choledocoduodenostomy and gastroenterostomy prior to the diagnosis of tuberculosis. He was admitted to hospital with moderately advanced, unilateral disease and multiple cavities, 1 to 2 cm. in greatest internal dimension. At that time his electrocardiogram revealed evidence of old anterior myocardial infarction. He was treated with the basic high dosage regimen to which was added para-aminosalicylic acid to elevate the serum level of isoniazid. Early during this hospitalization, he suffered another myocardial infarction, and was placed on strict bed rest. He complained bitterly of para-aminosalicylic acid gastrointestinal irritation, and was unable to accept this medication regularly even though it was needed to elevate the serum isoniazid level. Resection of the cavitary disease was considered necessary, but surgery was postponed indefinitely because of the cardiac status. The patient left the hospital, discontinued all drugs in the seventh month of chemotherapy, and six months later he was readmitted to our hospital excreting streptomycin-susceptible, para-aminosalicylic acid-susceptible, catalase-positive, isoniazid-resistant organisms in his sputum. To date this is the only known relapse among the 153 Group I patients.[3]

DISCUSSION

The results of serum assays for antimicrobially-active streptomycin and isoniazid have suggested that conventional dosage could not be relied upon to insure adequate delivery of sterilizing concentrations to susceptible tubercle bacilli in chemotherapy of pulmonary tuberculosis. Therefore, a chemotherapeutic regimen was designed combining high dosage of streptomycin with high dosage of isoniazid. This high dosage regimen was administered to all patients excreting streptomycin-susceptible, isoniazid-susceptible tubercle bacilli on admission. Six hour serum assays for streptomycin and isoniazid were performed on some of these patients in order to document the adequacy of these drugs.

ganisms from the failure were fully susceptible to 8 μg. per ml. even though highly resistant to 2 μg. SM per ml.

[3] Since this report was submitted for publication another patient has suffered a bacteriologic relapse. Both relapse cases had several points of similarity. Both became bacteriologically negative prior to the start of chemotherapy, even though positive on smear and culture on admission. Both left the hospital shortly after six months of chemotherapy, and both discontinued chemotherapy against medical advice in the seventh month. This second patient relapsed 21 months after stopping medications. *And, finally, these two relapses were the only patients in Group I who were treated with drugs for less than nine months.*

A few large groups have carried out extensive studies on the chemotherapy of pulmonary tuberculosis (18–21). Retrospective analysis of the results of these investigations of conventional drug treatment regimens has revealed that 10 to 20 per cent of previously untreated patients fail to achieve the state of bacteriologic negativity without collapse procedures or surgical resection. *In vitro* drug-susceptibility tests indicated that most of these failures of chemotherapy resulted from the emergence of drug-resistant mutant populations of tubercle bacilli. In spite of the fact that combined drug regimens were used in these studies, failure of combined chemotherapy employing conventional dosages frequently was manifested by the early emergence of mutants resistant to only one of the two drugs administered. Experiments performed in our laboratory have indicated that selection of mutants resistant to one drug on exposure to two drugs is a consequence of exposure to effective concentrations of that one drug plus ineffective (sub-inhibitory) concentrations of the companion drug; in effect, this is equivalent to exposure to only one drug although the single-drug-resistant mutants recovered are strikingly reduced in numbers (see Experiment No. 2, in the first report of this series).

It seemed reasonable to postulate that increasing the dosage of each drug should largely eliminate such evidences of inadequate delivery of drugs as the emergence of organisms resistant to one of the two drugs. Support for this hypothesis was found in the British Medical Research Council reports (18, 19) which indicated that streptomycin, 1.0 Gm. daily, combined with isoniazid, 200 mg. daily, gave results superior to streptomycin, 1.0 Gm. twice each week, plus the same dosage of isoniazid (200 mg. per day). Further support for this postulate was evident in a study by Biehl (22) who made a clinical comparison between streptomycin, 1.0 Gm. twice each week, combined with isoniazid, 5 mg. per Kg. per day, and the same dosage of streptomycin (1.0 Gm. twice each week) plus isoniazid, 20 mg. per Kg. per day. The regimen including high dosage isoniazid combined with low dosage streptomycin (2.0 Gm. per week) failed to prevent the emergence of isoniazid-resistant organisms. Viewed in the light of these studies, the excellent results obtained in Group I patients could have been anticipated.

The results obtained by application of the high dosage isoniazid-high dosage streptomycin basic regimen to the chemotherapy of Group I patients are superior to the results reported by other investigators with the exception of Ross, Horne, Grant and Crofton (23, 24). It can be contended that this superiority is attributable to factors other than chemotherapeutic. The type of patient material in terms of anatomic extent of disease, number and size of cavities, and duration of active disease, for example, may be less challenging among patients accepted for admission to National Jewish Hospital. There may be other important variables, such as altitude (5,300 ft.), or the complex advantages of hospitalization *per se* which would favor the obtaining of such results. However, our experience prior to October of 1954 with chemotherapeutic regimens which did not include both high dosage of isoniazid and high dosage of streptomycin provided adequate evidence, in our opinion, that such unidentified variables were not operating in these studies. For example, between January of 1953 and October of 1954, 43 adult patients excreting typical tubercle bacilli susceptible to both streptomycin and isoniazid were treated with streptomycin, 1.0 Gm. intramuscularly every third day, combined with isoniazid, 8 or more mg. per Kg. per day, for at least four months before collapse procedures or surgical resection. Thirty-one (72 per cent) of these patients became bacteriologically negative by Day 120 of chemotherapy, but 11 (26 per cent) were chemotherapeutic failures by virtue of the emergence of isoniazid-resistant organisms (25). Thus, our experiences at this institution prior to the initiation of the high dosage regimen described here were quite comparable to the experiences of other investigators employing similar chemotherapeutic regimens.

With regard to the results reported by Crofton and his associates, it should be pointed out that two uncontrolled variables render their studies incomparable with the Group I experiences described here. First, they have reported that all three drugs (streptomycin, isoniazid and para-aminosalicylic acid) were often used during the first six to eight weeks of chemotherapy, *i.e.*, until the results of drug-susceptibility studies became available (26). Para-aminosalicylic acid is known to decrease the rate of metabolic inactivation of iso-

niazid (2, 3), and, therefore, can obscure the role played by this phenomenon in determining the effectiveness of chemotherapy with isoniazid. Second, other therapeutic measures were taken (pneumoperitoneum and so forth) *ad lib.*, in addition to chemotherapy, before four months after bacteriologic conversion of the sputum to negative.

The poor results of antimicrobial therapy alone in patients of Group III were not surprising (Table I). They point up the need for additional effective chemotherapeutic agents for tuberculosis. On the other hand, the 47 per cent conversion rate (16 patients converted out of 34 treated for nine to 12 months without collapse procedures or surgery) for patients in Group II may seem unexpectedly low. However, many of the patients in this group were excreting populations of tubercle bacilli which were resistant to para-aminosalicylic acid as well as to either streptomycin or isoniazid on admission to hospital. Therefore, in effect, several of these patients received the benefit of only single-drug treatment during the period of this study.

It has been customary for some clinicians to reserve "one good drug" for use at the time of surgery. These results indicate that it would be better to strive for optimal chemotherapeutic results from the initiation of treatment.

The success observed on application of the appropriate basic high dosage regimen in patients excreting drug-susceptible tubercle bacilli seems to eliminate any doubt as to the clinical importance of delivering adequate concentrations of at least two antimicrobial drugs *in vivo*. As was implied in the first report of this series (1), there is a definite, but very low, incidence of mutant forms of tubercle bacilli which are simultaneously resistant to both streptomycin and isoniazid. Thus, there will be a definite incidence of chemotherapeutic failure on any two-drug regimen. Just as adequate two-drug regimens markedly decrease the incidence of chemotherapeutic failure manifested by the emergence of one-drug-resistant mutant populations, so should adequate regimens of three or more drugs decrease the incidence of chemotherapeutic failure manifested by the emergence of two-drug-resistant mutant populations. It seems fair to assume that the one instance of double mutation to resistance to both isoniazid and streptomycin which occurred in these studies could have been prevented had a third drug, *i.e.*, para-aminosalicylic acid, been added to his therapeutic regimen before the twelfth week of chemotherapy.

The present study constitutes a relatively short-term evaluation of chemotherapy of pulmonary tuberculosis in adults. Resting bacterial cells which escape sterilization by virtue of being physiologically insusceptible to the action of streptomycin or isoniazid remain a threat able to initiate an exacerbation of the disease months or even years later (27). Therefore, final evaluation of any approach to the chemotherapy of tuberculosis requires a long period of follow-up observation.

Among 133 patients in Group I who presented roentgenographic evidence of cavitary disease on initiation of chemotherapy and who remained in hospital at least four months after the date of bacteriologic conversion to negative, 66 (50 per cent) had persistence of cavitation ("open cavity healing" by clinical criteria). This is the highest incidence of "open cavity healing" reported in the literature. It can fairly be attributed to the avoidance of those measures—strict bed rest, and pulmonary collapse procedures—which, in our opinion (28), are in serious conflict with the purposes of appropriate and effective antimicrobial chemotherapy.

For patients treated with conventional drug regimens plus bed rest, important unfavorable prognostic significance has been attached to the persistence of roentgenographically-visible cavitation even when it is accompanied by the absence of acid-fast bacilli from the sputum (29). On the other hand, more recently, evidence has been presented by some investigators (30, 31) that the association of open, air-filled cavities with persistently negative sputum bacteriology after more effective protocols of chemotherapy does not have unfavorable significance. It will be interesting to determine whether patients treated under the high dosage combined drug regimen will show relapse rates lower than those which have been observed on previous modes of therapy. Results obtained from bacteriological studies on surgically resected pulmonary lesions from patients in Group I of this study provide corroborative evidence that this may, indeed, be anticipated (32).

In retrospect, the observations on the variations in the metabolic disposition of isoniazid and on the six hour serum levels after streptomycin, 20

mg. per Kg., indicate that many of the Group I patients might have responded as favorably to chemotherapy employing lower dosages of these drugs. Indeed, in view of the significant incidence of drug reactions (12 per cent) in our experience with the high dosage regimen for all patients, it seems that routine high dosage imposes an unnecessary risk. Thus, the best approach should be one in which dosages of appropriate drugs are adjusted according to the results of specific pharmacologic assays.

The minimal immediate chemotherapeutic failure and the low early relapse rate in Group I patients, on the one hand, and the high failure rate in the Group II and Group III patients, on the other hand, provide evidence of the clinical value of the drug-susceptibility tests employed in these studies for predicting response to chemotherapy with these drugs.

Ninety-nine per cent of the patients who excreted streptomycin-susceptible, isoniazid-susceptible tubercle bacilli at the start of chemotherapy at this hospital achieved sputum conversion on the high dosage regimen without collapse or surgery, or strict bed rest. Microbiologic serum assay allowed prompt discovery and correction of isoniazid dosages in those few individuals who might have had inadequate drug with the unmodified regimen. The general principles of primary chemotherapy of tuberculosis upon which the treatment of the Group I patients was based have been outlined previously (28). The very low chemotherapeutic failure rate observed in such patients in this study confirms our previous opinion (28) that the drug-treatment failures of conventional chemotherapy are largely due to inadequate dosage of drugs or indiscriminate combinations of therapeutic measures.

SUMMARY

Three hundred eighteen adult patients with bacteriologically proved pulmonary tuberculosis due to typical tubercle bacilli were admitted consecutively to the National Jewish Hospital at Denver between October 1, 1954, and June 1, 1957, and were treated for at least six months. Of these, 153 were excreting organisms which were susceptible to both streptomycin and isoniazid. These patients were subjected to a high dosage streptomycin, high dosage isoniazid chemotherapeutic regimen. The sputa of 152 of the patients excreting populations of tubercle bacilli which were streptomycin- and isoniazid-susceptible became bacteriologically converted to negative by the end of the sixth month of chemotherapy. None of these 152 patients excreted drug-resistant organisms prior to conversion. There was one early chemotherapeutic failure, as manifested by the failure to achieve conversion and by the emergence of double-drug-resistant mutants. There has also been one bacteriologic and roentgenographic relapse.

Two other groups of patients, those who were already excreting drug-resistant organisms on admission to hospital, were studied for comparison: those excreting tubercle bacilli resistant to one drug, and those with organisms resistant to two drugs. Neither group demonstrated impressive response to chemotherapy. These results established the value of the special drug-susceptibility testing techniques employed for predicting the clinical response to treatment with streptomycin or isoniazid.

The theoretical and practical consequences of these studies were discussed.

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COMBINED DRUG TREATMENT OF TUBERCULOSIS. IV. BACTERIOLOGIC STUDIES ON THE SPUTUM AND RESECTED PULMONARY LESIONS OF TUBERCULOUS PATIENTS

By ANGELINE D. HEATON, WILLIAM F. RUSSELL, JR., JOHN DENST AND GARDNER MIDDLEBROOK

(From the Department of Research and Laboratories, National Jewish Hospital at Denver, and the Departments of Pathology and Microbiology, University of Colorado School of Medicine, Denver, Colo.)

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The altered response to antituberculous agents of both the tubercle bacilli cultured from the sputum and from excised pulmonary lesions during and after therapy with these drugs has aroused the interest of the bacteriologist, the pathologist and the clinician. Therefore, many studies of this problem based upon clinical material subjected to wide varieties of drug-treatment regimens have been reported. These investigations have indicated that a significant proportion of clinically inactive lesions resected from drug-treated, sputum-negative patients still contain viable tubercle bacilli (1–7). For this reason surgical excision of roentgenographically demonstrable residual lesions is widely practiced.

The purpose of this paper is to present a comparison of the preoperative and postoperative sputum results, including drug-susceptibility testing, with similar bacteriologic studies carried out on the specimens of pulmonary tissue removed at surgery from 220 tuberculous patients. These bacteriologic findings have also been correlated with the drug-treatment regimens used at National Jewish Hospital before and after October 1, 1954, including the results of bacteriologic examination of resected pulmonary lesions from 37 patients who had been originally treated with the high dosage combined-drug regimen described in the third paper of this series (8).

MATERIAL AND METHODS

A. Examination of sputum. Digestion of the sputum (10 ml. or less) was performed by addition of "activated pangestin" [1] (Difco), 1.0 ml., and sodium bicarbonate 10 per cent, 1.0 ml. The specimen was diluted with distilled water to 20 ml., and incubated in a water bath at 40° C. for 30 minutes. Following this, "activated pangestin," 1.0 ml., was again added and the specimen reincubated in the same manner after dilution with distilled water to about 45 ml.; the specimen was centrifuged at 2,500 rpm for 30 minutes. The supernatant fluid was discarded, and a smear of the concentrate was made and stained by the Ziehl-Neelsen technique. Two hundred oil immersion fields were examined for acid-fast rods.

Specimens were divided into three groups according to the number of acid-fast rods (AFB) seen per oil immersion field: I. Containing < 1 AFB per oil immersion field. II. Containing 1-10 AFB per oil immersion field. III. Containing > 10 AFB per oil immersion field.

During examination of the stained films from the concentrate, further digestion and decontamination with weak alkali was performed, as described elsewhere (9).

Sediments from specimens of Group I were inoculated undiluted and at 10^{-1} dilution; Group II, 10^{-1} and 10^{-2}; Group III, 10^{-2} and 10^{-3}.

In each case, after dilution, approximately 0.1 ml. was inoculated directly onto each of four quadrants of a Felsen plate which contained the 7H-10 solid agar medium plus 1.0 μg. of malachite green per ml. (9). The quadrants were as follows: Quadrant 1, no drug (control); Quadrant 2, 2.0 μg. streptomycin per ml.; Quadrant 3, 0.2 μg. isoniazid per ml.; and Quadrant 4, 2.0 μg. para-aminosalicylic acid per ml. This provided direct drug-susceptibility testing, as well as routine culture, on every sputum specimen.[2] The definition of drug-susceptibility employed in this report is: A strain or population of tubercle bacilli is susceptible to the action of streptomycin or isoniazid if *less than* 1 per cent of those

[1] Pangestin (Difco) is activated in the following manner: Dissolve-suspend one part of "pangestin" in 25 parts of distilled water; centrifuge at high speed in the angle centrifuge in the cold, pour off the clear, amber-colored supernate. Mix this solution with an equal volume of a 12 per cent aqueous solution of magnesium sulfate.

[2] Prior to 1955, this last quadrant was used to test 10 μg. isoniazid per ml. During part of 1955, this quadrant was used to test pyrazinamide, 50 μg. per ml. Since that time, para-aminosalicylic acid has been used in this quadrant. Therefore, the results of susceptibility to para-aminosalicyclic acid (PAS) test are not reported here.

members yielding colonies on the control are able to proliferate on culture media containing either 0.2 μg. of isoniazid per ml. or 2.0 μg. of streptomycin per ml. Similarly, any tubercle bacilli which multiply on media containing these concentrations of drugs, are drug-resistant, whether they derive directly from a wild population or are members of a population which has been selected by exposure to either of the drugs.

B. Examination of tissue. Immediately following surgical resection, each specimen was subjected to pathological examination in the fresh state.[3] The preoperative roentgenograms were correlated with the findings of the resected specimen. Different sets of sterile instruments were used for obtaining bacteriologic specimens from each of the lesions so that the possibility of cross-contamination was minimized. Caseous lesions which measured less than 3 mm. in diameter were not studied individually, but were sometimes included with a predominantly fibrous lesion for culture. Impression smears were made from the necrotic material of the lesion. Abundant necrotic tissue which included a portion of the wall of the lesion was excised for culturing. When small lesions were encountered, the major portion was so used. The relationship of the lesion to the bronchi was then determined. The remaining pulmonary tissue was processed for histologic study.

Each bacteriological specimen was macerated with sterile dissecting scissors and sterile forceps, and placed in a test tube with 5 ml. of filter-sterilized 0.2 per cent bovine serum albumin in distilled water and further macerated in a Teflon-glass grinder (11). The Felsen plates used for this study were prepared in the same manner as those used for culture of the sputum, and contained the same concentrations of drugs. Each of the four quadrants of a Felsen plate, containing the 7H-10 agar medium with or without drugs, was inoculated with 0.1 ml. of this suspension. Similarly, a 10^{-4} dilution was made and inoculated onto the quadrants of a second Felsen plate.

Both sputum and tissue cultures were incubated for 21 days at 36° C. under an atmosphere of 2 to 5 per cent CO_2 in air. Some authors have emphasized the importance of keeping cultures of surgical specimens over very long periods of time in order to be certain that colonies of tubercle bacilli (especially isoniazid-resistant organisms) would not appear at a later date (12). However, evidence has been presented elsewhere that the culture medium and techniques employed in the studies described here give a reliable index of the viable bacterial count within three weeks (9). Cultures of several of the surgical specimens described here which were smear-positive and culture-negative at 21 days were further incubated for long periods of time (up to 11 months). None of these yielded colonies.

Determinations of catalase activity as a routine procedure on cultures of tubercle bacilli recovered from resected specimens was begun on May 1, 1956. These determinations were done as previously described (13).

[3] Between 10-1-54 and 6-15-56, surgical specimens were submitted to deep freezing prior to bacteriological study, according to the method previously described by Hobby and co-workers (10).

DEFINITIONS

A. Histopathologic terms. The lesions were classified as open cavities, "filled" cavities, nodules and lung parenchyma (predominantly scar tissue).

Necrotic lesions which apparently at one time had been open and possessed a communicating bronchus, but which did not exhibit an empty space, were classified as "filled" cavities. Localized, gross areas of tuberculous tissue, regardless of their physical state, with no evident bronchocavitary communication were classified as nodules. Fibrous or scar tissue, occasionally with minute sclerotic foci, was classified as lung parenchyma.

It is important to emphasize that classification of a cavity as "filled" does not necessarily imply that the cavity was functionally closed.

B. Bacteriologic conversion of the sputum. For the purpose of this communication, sputum conversion to the bacteriologically negative state is defined to have occurred when both negative smears and negative cultures have been obtained on at least three consecutive monthly sputa with no subsequent positives. The date of conversion is defined as the date of the first of such a series of negative smears and cultures.

CLINICAL MATERIAL

Bacteriologic study, including drug-susceptibility testing, was performed on 298 individual lesions in 229 consecutive surgically resected specimens from 220 patients between October 1, 1954, and October 1, 1957.

Beginning on October 1, 1954, the application of high dosage streptomycin (20 mg. per Kg. per day) in combination with high dosage isoniazid (16 mg. or more per Kg. per day) together with pyridoxine, 50 to 100 mg. daily, was used in the treatment of all patients excreting typical tubercle bacilli susceptible to these drugs. Para-aminosalicylic acid was added when indicated to elevate the isoniazid levels. This approach to chemotherapy of pulmonary tuberculosis has been described in detail in the third paper of this series (8). Prior to that time, conventional dosages of streptomycin (1 Gm. every three days) combined with intermediate to high doses of isoniazid (8 mg. or more per Kg. per day) were used.

Original treatment cases are defined as those who received three weeks or less of chemotherapy with streptomycin, isoniazid or para-aminosalicylic acid, either singly or in combination, prior to admission. There was a total of 73 patients considered as original treatment cases: 24 admitted prior to October 1, 1954, and 49 admitted after October 1, 1954. Populations of tubercle bacilli isolated from the sputa of these 73 original treatment patients were both isoniazid- and streptomycin-susceptible at the time of admission. Six of the nine original treatment patients whose chemotherapy dated from before October of 1954 were excreting isoniazid-resistant tubercle bacilli at surgery. The remaining three

TABLE I

Summary of 200 patients according to status of chemotherapy on admission and bacteriologic status of sputum prior to resection

Preoperative bacteriologic status	Original treatment		Retreatment	Total
	Before 10–1–54	After 10–1–54		
Positive	9*	2†	96	107
Converted	15‡	47§	51‖	113
Total	24	49	147	220

* Six of these were excreting isoniazid-resistant organisms at surgery. The remaining three were sputum-negative but not converted as defined.

† These two patients were negative but not converted at surgery.

‡ One lesion of one patient was culture-positive.

§ All resected specimens from these 47 patients yielded negative cultures.

‖ Lesions from four patients were culture-positive.

plus the two positives treated after October of 1954 were bacteriologically negative but not converted, as defined.

Retreatment cases are defined as those patients who had received antituberculous chemotherapy for more than three weeks prior to admission. One hundred and forty-seven patients comprised this group. Ninety-six patients were excreting drug-resistant populations of tubercle bacilli on admission. And, 33 patients were either bacteriologically negative or yielded insufficient numbers of colonies of tubercle bacilli to permit satisfactory drug-susceptibility testing (Tables I and II).

RESULTS

At the time of surgical resection 107 patients were not "sputum converted": Eleven of them belonged to the group of 73 original treatment patients (15 per cent) and 96 belonged to the group of 147 retreatment patients (65 per cent). The remaining 113 were sputum-negative and "converted." Sixty-two patients in this group were first treated with antimicrobial agents at the National Jewish Hospital (85 per cent of the total number of original treatment patients) and 51 belonged to the "retreatment" group (35 per cent of the total number in this group).

Among the 51 "retreatment" patients who were converted before the time of surgical resection, 33 either had been sputum-negative since admission to hospital or were excreting very few organisms in the sputum. None were excreting populations of tubercle bacilli which were susceptible to streptomycin and isoniazid at the time when they were transferred to higher dosage combined-drug treatment (streptomycin and isoniazid). And the remaining nine patients represented 8.5 per cent of 105 retreatment patients who were excreting drug-resistant populations of tubercle bacilli on admission to hospital (Table II).

It is also shown in Table I that 15 (60 per cent) of 24 original treatment patients admitted prior to October 1, 1954, were sputum-converted before surgical resection, while 47 (96 per cent) of 49 original treatment patients admitted after that date, and treated with high dosage of both streptomycin and isoniazid, were sputum-converted before surgical resection.

Of the 298 lesions studied, 144 (48 per cent) were positive on culture; three-fourths of these were cavitary lesions. Of the remaining 154 culture-negative lesions, one-half were positive on smear. The number of solid lesions in this culture-negative group was twice as great as the number of cavitary lesions (Table III).

In 206 out of the total of 220 patients, there was complete agreement in the bacteriologic results of the study of the preoperative sputum specimens and of the resected specimens. Anomalous

TABLE II

Bacteriological status of 147 retreatment cases at the time of admission and at resection

Bacteriologic status on admission	Bacteriologic status at time of surgery		Per cent converted	Total patients
	Positive	Converted		
Positive and drug resistant	96	9	9	105
Positive and drug susceptible	0	9	100	9
Insufficient growth for testing, or sputum negative*	0	33	100	33
Total	96	51		147

* Sputum-negative or too few colonies grew for reliable drug-susceptibility testing.

TABLE III

Two hundred ninety-eight lesions from 220 patients according to type and bacteriology of lesion

Type of lesion	Bacteriology			Total
	Z.-N. 0* cult. 0	Z.-N. + cult. 0	Z.-N. + or 0 cult. +	
Cavitary†	35	27	116	178
Solid	44	48	28	120
Total	79	75	144	298

* Z.-N. = Ziehl-Neelsen stain of impression smear.

† Both "open" and "filled" cavities.

TABLE IV

Bacteriologic findings of resected lesions according to preoperative sputum status (220 patients)

Sputum status before surgery	Z.-N. 0 cult. 0	Z.-N. + cult. 0	Z.-N. + or 0 cult. +	Total
Positive	5*	4*	98	107
Converted	54	54	5*	113
Total	59	58	103	220

* Anomalous results (see text, Discussion).

results were obtained in 14 (7 per cent) of the cases (Table IV).

The nine patients classified as sputum-positive prior to surgery, but whose tissue failed to yield tubercle bacilli on culture, have been analyzed (Table IV). Two patients, consistently sputum-positive, yielded a solid lesion and an open cavity; both patients remained sputum-positive after surgery. It is quite clear the lesions responsible for the positive sputa were not resected. Two patients, consistently sputum-negative for five months, had one positive sputum immediately prior to surgery. Smear positive solid lesions were encountered in each case. Such observations are consistent with those of Canetti (14), who reports that solid caseous lesions may contain numerous visible bacilli while few and sometimes none of them may be viable. Five patients had three consecutive negative sputa prior to surgery, but had not been negative for the full period of three months. These might have been defined as "converted" had surgery been delayed.

All patients except the first two remained sputum-negative following surgery. Thus, in reality, only two of the nine patients definitely yielded anomalously culture-negative tissue, a 2 per cent disagreement between sputum and resected tissue bacteriology among 107 sputum-positive patients.

The five patients who were negative prior to resection, but whose pathologic specimens were positive on culture (Table IV), were originally treated with conventional dosage regimens of streptomycin and isoniazid at National Jewish Hospital prior to October, 1954, or at other institutions (Table I). Three of these specimens contained open cavities and a fourth specimen contained a "filled" cavity. Streptomycin-susceptible, isoniazid-resistant tubercle bacilli were cultured from each specimen. The fifth patient had never been sputum-positive at this hospital, but aspiration of a large pleural effusion yielded streptomycin-susceptible, isoniazid-susceptible tubercle bacilli on culture. A sub-pleural nodule in the resected lung yielded organisms with drug-susceptibility characteristics identical to those cultured from the fluid. All five patients remained sputum-negative after surgery.

TABLE V

Bacteriologic findings—139 resected lesions from 113 sputum converted patients

Lesions	Z.-N. 0 cult. 0	Z.-N. + cult. 0	Z.-N. + or 0 cult. +	Total patients
Open cavity	31	12	3	46
"Filled" cavity	0	9	1	10
Nodule	26	40	0	66
Lung parenchyma*	13	3	1	17
Total	70	64	5	139

* Fibrous tissue occasionally containing minute sclerotic foci.

The 139 lesions from the 113 converted cases are analyzed in Table V. All but three of the 46 open cavities were negative on culture and three-fourths of them were negative on smear as well. On the other hand, nine of the 10 "filled" cavities were positive on smear and negative on culture; one was positive on culture. No "filled" cavities were negative on smear and culture. Although there were no culture-positive nodular lesions in this group, about two-thirds of them were positive on smear.

In marked contrast to these results are the 159 lesions studied from 107 sputum-positive patients (Table VI). One hundred and two of 104 open cavities were positive on culture. Ten of 18 "filled" cavities were positive on culture, and six

TABLE VI

Bacteriologic findings—159 resected lesions from 107 sputum-positive patients

Lesion	Z.-N. 0 cult. 0	Z.-N. + cult. 0	Z.-N. + or 0 cult. +	Total
Open cavity	2	0	102	104
"Filled" cavity	2	6	10	18
Nodule	4	3	21	28
Lung parenchyma*	3	0	6	9
Total	11	9	139	159

* Fibrous tissue occasionally containing minute sclerotic foci.

of the remaining eight were positive on smear. Seventy-five per cent of 28 nodular lesions were positive on culture, and three of the remaining seven were positive on smear.

Analysis of the drug-susceptibility patterns of the populations of tubercle bacilli recovered from sputum of unconverted patients and from their resected specimens is presented in Table VII. Identical patterns of drug susceptibility of populations of tubercle bacilli recovered from the sputa and resected lesions were found in 94 patients. There were two patients whose surgical specimens yielded anomalous results. Apparently one of these patients was excreting tubercle bacilli from two foci. Prior to resection 22 consecutive highly positive sputum specimens had been obtained from this patient. The bacterial populations in 18 of these specimens were resistant to isoniazid and resistant to 2 μg. streptomycin per ml. The remaining four specimens contained organisms resistant to isoniazid, but completely susceptible to streptomycin. The resected specimen contained a "filled" cavity with isoniazid-resistant, streptomycin-susceptible organisms. After resection the sputum remained positive and contained only isoniazid-resistant and streptomycin-resistant tubercle bacilli. The preoperative sputum of the second patient contained tubercle bacilli resistant both to streptomycin and to isoniazid. Streptomycin-susceptible, isoniazid-resistant tubercle bacilli were cultured from a resected open cavity. Apparently this lesion was the source of the highly positive preoperative sputum since the patient became sputum-negative immediately after surgery. No explanation of this discrepancy is evident.

Catalase activity was determined on tubercle bacilli recovered from positive cultures from the surgical specimens and from the preoperative sputum of 54 patients (Table VIII). The results indicate a high degree of correlation. Photochromogenic or "yellow" bacilli were recovered from the preoperative sputa and resected specimens of four patients. Of the remaining 50 patients who had been excreting typical tubercle bacilli, there were two whose organisms from the surgical specimen showed somewhat less catalase activity than did the organisms recovered from the preoperative sputum.

TABLE VII

Comparative results of drug-susceptibility testing tubercle bacilli from preoperative sputum and resected lesions (96 patients)

Source	SM-S* INH-S†	SM-R INH-S	SM-S INH-R	SM-R INH-R	Total patients
Sputum	0	2	16	78	96
Lesions	0	2	18	76	96

* SM-S (or R) = streptomycin-susceptible (-resistant).
† INH-S (or R) = isoniazid-susceptible (-resistant).

TABLE VIII

Comparative results of catalase testing tubercle bacilli from preoperative sputum and resected lesions (54 patients)

Source	Catalase activity: High* +++++	Moderate† ++ to +++	Reduced or absent 0 to +	Total patients
Sputum	4	9	41	54
Lesions	4	7	43	54

* Photochromogenic "yellow" bacilli.
† Typical isoniazid-susceptible tubercle bacilli.

DISCUSSION

Numerous studies have been done to correlate the bacteriology of resected tuberculous tissue with the various chemotherapeutic regimens, the duration of chemotherapy, the incidence and duration of sputum conversion prior to resection, and the effect of chemotherapy upon the type and the size of the lesion. Results of these studies have indicated that the longer the duration of chemotherapy and of sputum conversion prior to resection, the lower the incidence of viable organisms recovered from lesions resected from sputum converted patients. These studies have also indicated that there was a higher incidence of viable organisms recovered from open cavitary lesions of sputum-negative patients.

The results of our study generally confirm these findings with the exception that the recovery of viable organisms in the lesions of sputum converted patients was very low in our series. The most striking difference between our results and those of other investigators in this field (1–7, 10, 14) is our failure to recover viable organisms from the open cavitary lesions resected from sputum-negative patients treated with a high dosage combined-drug regimen of streptomycin and isoniazid. It has been suggested (15, 16) that for permanent arrest of tuberculous infection a re-

sidual open cavitary lesion may be a more desirable end result of chemotherapy than a "filled" cavity. This is supported by the material presented in Table V. Not one of the 10 filled cavities removed from sputum-negative patients was negative on both smear and culture. On the other hand, 65 per cent of the 46 open cavities resected from this group of patients were negative on both smear and culture and only three of these lesions yielded positive cultures, all from patients originally treated with conventional as opposed to high dosage, combined-drug regimens.

The question may be raised as to whether the bacteriologic methods employed in this study are as effective in isolating small numbers of tubercle bacilli as those employed by other investigators. However, the percentage of negative cultures on surgical specimens, obtained from patients whose preoperative sputum specimens were definitely positive, was very low in this study, about 2 per cent—indeed, as low as any recorded in the literature. Furthermore, broader evaluation of the bacteriologic methods employed in this study has revealed them to be at least as efficient in isolating tubercle bacilli from the sputum as any of the more conventional methods (9).

Middlebrook and Cohn (9) have reported that 26 per cent of patients at National Jewish Hospital with bacteriologically proven tuberculosis never had positive concentrate smears of the sputum or gastric contents. They have also reported that less than 3 per cent of those sputum specimens with positive smears yielded negative cultures (including contaminated cultures) in our laboratories. These experiences speak for a very high degree of effectiveness of the methods employed in this study for cultivating small numbers of tubercle bacilli.

The incidence of positive cultures of resected pulmonary tissue from sputum-negative patients accumulated in the literature as well as in our series is presented in Table IX. It is evident that our series presented the lowest incidence (5 per cent) of positive cultures from sputum-negative patients.

There was a high degree of correlation (98 per cent) between the drug susceptibilities of tubercle bacilli recovered from the culture of the resected specimen, on the one hand, and of the tubercle bacilli recovered from the preoperative sputum, on the other. This may be contrasted with the results of a similar study by Kennedy, Vandeviere, Melvin and Willis (17), who reported only 67 per cent agreement.

TABLE IX

Reported incidence of positive cultures of resected pulmonary tissue from sputum-negative patients

Ref. no.	Investigators	No. of patients	Total positive
			%
(1)	Hurford and Valentine (1957)*	50	38
(2)	Wayne and Salkin (1956)	201	15
(3)	Bell (1956)	147	40
(5)	Stewart *et al.* (1956)‡	47§	23
(7)	Adler *et al.* (1955)	103§	18
(8)	National Jewish Hospital	113	5

* Only closed lesions reported.
† Only open cavities reported.
‡ Only lesions yielding drug-susceptible organisms included.
§ Probably not all patients sputum-negative at the time of surgery.

There was also a high degree of correlation between the results of catalase tests on cultures of tubercle bacilli from sputum and from resected specimens (18). The finding of a high percentage of culture-positive sputa and lung lesions yielding only isoniazid-resistant catalase-diminished or catalase-negative mutants of tubercle bacilli is not surprising, since nearly all patients in this study had been treated at this institution with at least 8 mg. isoniazid per Kg. per day for several months prior to surgical resection (19). This was done regardless of the results of isoniazid-susceptibility tests, for reasons presented elsewhere (20).

One-quarter of the original treatment patients whose chemotherapy was begun prior to October 1, 1954, were chemotherapeutic failures excreting isoniazid-resistant organisms before the time of resection. Although they were originally treated with isoniazid, 8 or more mg. per Kg. per day, streptomycin was not given on the highly effective dosage schedule of 1 to 2 Gm. daily for 90 days or more later employed routinely at this institution, as described elsewhere (8). In contrast to the bacteriologic results obtained in these patients were those obtained in the 49 patients who received their original chemotherapy at National Jewish Hospital after October 1, 1954, with high doses of daily streptomycin and isoniazid (Table I).

The two patients in this last group who were sputum-positive at the time of resection required

emergency thoracotomy early during their original course of chemotherapy. The remaining 47 patients were all converted on chemotherapy alone, and without bed rest, prior to resection. All these patients, while they were still positive, continued to excrete only streptomycin-, isoniazid-susceptible tubercle bacilli, though in progressively diminishing numbers, until the time of sputum conversion. No positive tissue cultures were obtained from the resected tissues. The postoperative follow-up period on these patients ranges from eight months to more than two and one-half years, without evidence of roentgenographic or bacteriologic relapse in any of these individuals.

Thirty-seven of these 49 patients had moderately advanced or far advanced bilateral disease with cavity size ranging from 1.5 cm. to greater than 7 cm. They belong to a special study [part of Group I described in the third paper of this series (8)]. The duration of chemotherapy prior to resection in these patients was less than 12 months, an average of 209 days, ranging from 159 to 353 days, but longer than four months. Our results in this group of cases are in disagreement with those of other investigators who suggest that 12 months of combined chemotherapy are probably necessary to assure negative cultures on surgical specimens from such cases (5).

The discrepancy between our results and the results of other investigators may, indeed, be due to important differences in the dosage of drugs employed (3 to 5 mg. of isoniazid per Kg. per day, and streptomycin, 40 to 60 mg. per Kg. [2 to 3 Gm.] per week in most other studies, in contrast with isoniazid, 16 mg. per Kg. per day, and streptomycin, 140 mg. per Kg. per week, for these patients). The earliest bacteriologic and pathologic evidence of the superiority of the use of 7 over 2 or 3 Gm. streptomycin per week in combined drug therapy was reported by D'Esopo and co-workers (6). The lowest incidence of positive cultures of resected tissue from sputum converted patients in their studies was obtained when streptomycin was used in dosage of 1 Gm. daily with para-aminosalicylic acid.

In view of the low incidence of positive cultures from the resected specimens of patients rendered sputum-negative by intensive combined drug treatment, doubt may be raised as to what purpose is served by surgical resection of residual pulmonary lesions of such patients (21). Nevertheless, even if it is to be assumed that the culture techniques employed in this study were perfectly effective in detecting any viable tubercle bacilli, it must be emphasized that only a small sample of each lesion was actually submitted to culture. Therefore, it would seem unwise to take a dogmatic position on this issue in the current state of our knowledge.

SUMMARY

1. Bacteriologic studies on the preoperative sputum and on individual pulmonary lesions of 220 patients subjected to resection for pulmonary tuberculosis have been analyzed. Two hundred six (93 per cent) of these were in agreement in that the sputum and the surgical specimens were either both negative on culture or both positive on culture.

2. Discrepancy between the results of drug-susceptibility studies performed on the preoperative sputa and on the resected tissue specimens was found in two of 96 patients.

3. There was a high degree of correlation (95 per cent) between the catalase activity of organisms recovered from preoperative sputa and the catalase activity of tubercle bacilli recovered on culture of resected specimens.

4. There was a low incidence (10 per cent) of sputum conversion on chemotherapy among the retreatment cases excreting tubercle bacilli resistant to streptomycin or isoniazid, or to both agents.

5. The high incidence of bacteriologically negative results on surgically resected specimens from sputum-negative patients originally on high dosage combined-drug treatment with isoniazid and streptomycin corroborates other evidence for the effectiveness of this approach to the chemotherapy of pulmonary tuberculosis.

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DIETARY FATS AND THE DIURNAL SERUM TRIGLYCERIDE LEVELS IN MAN

By PETER T. KUO* AND JOHN C. CARSON†

(From the Edward B. Robinette Foundation, Medical Clinics, Hospital of the University of Pennsylvania, Philadelphia, Pa.)

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A large number of studies have been made in the attempt to correlate serum cholesterol concentrations in man and in animals with varying amounts and with different kinds of dietary fat. However, only a few observations limited to the effect of diet upon the fasting human serum triglyceride concentration have been reported (1–4). This may be due, at least in part, to the high lability of this serum lipid fraction.

The works of Cunningham and Peters (5), Man and Gildea (6), Elkes, Frazer and Stewart (7), Brun (8), Turner and Steiner (9) and Havel (10, 11) clearly demonstrated that variations in both the fat and carbohydrate contents of each meal consistently produced rapid and marked changes in the triglyceride concentration in the human serum, while at the same time, they caused little or no change in the serum cholesterol concentration. Hence, in order to obtain a complete picture of the serum triglyceride level of a given subject, determination of its diurnal variations as well as the fasting level is desirable.

The present investigation is designed to study and compare the 24 hour serum triglyceride curves of the same individual after he is stabilized sequentially on an isocaloric: *a*) full diet which contains 100 to 140 Gm. of animal fats per day, *b*) rice and fruit diet, and *c*) corn oil formula diet in which corn oil constitutes 50 to 70 per cent of the total daily caloric intake of the individual.

MATERIAL AND METHOD

Clinical data of patients studied. Ten cooperative ambulatory subjects, seven males and three females, with various serum lipid concentrations were selected for the study. The clinical data and fasting serum triglyceride concentrations of these 10 subjects are summarized in Table I. In order to facilitate presentation of the data, the subjects in this series were classified into four groups according to their serum lipid concentration. Group I is made up of four subjects with normal serum lipid concentrations and showing no clinical evidence of coronary or peripheral arterial disease. In this group M.G. (female) had compensated rheumatic valvular heart disease, and A.P. (female) had lupus erythematosus and pseudobulbar palsy. K.B. and J.J. were two young healthy, male, medical students. In the "pure" hypercholesteremia group, (Group II) were R.J. and J.H. Their total serum cholesterol concentrations were at the 387 to 536 mg. per 100 ml. range, but their serum triglyceride concentrations were low. They both had large xanthalesmata on the eyelids and frequent attacks of angina pectoris. Patients R.K. and R.Ka. were classified in Group III. They had hypercholesteremia and mild degrees of hyperlipemia. Their total serum cholesterol concentrations were in the ranges of 950 to 1,200 and 310 to 356 mg. per 100 ml., respectively. R.K. has large xanthomata on the skin and tendons. R.Ka. had an acute attack of myocardial infarction at the age of 40 and had since been suffering from angina pectoris. Two male patients (J.H. and M.R.) with essential hyperlipemia were placed in Group IV. Both patients had clinical evidence of severe arterial insufficiency of the lower extremities.

Diets and blood samples. The total daily caloric intake of each subject while on full diet was estimated from the history. The full diet was estimated to contain 100 to 140 Gm. of animal fat (constituting 40 to 48 per cent of the person's total caloric intake) and 95 to 110 Gm. of proteins per day. Thereafter, the patient was allowed to continue for another seven to 10 days on the original diet, with instructions from the dietitian to keep the daily total caloric and fat intake constant and to eat the meals at 8:00 a.m., 12:00 noon and 6:00 p.m. To obtain the first set of diurnal serum triglyceride and other serum lipid curves, starting at 8:00 a.m., venous blood samples were drawn immediately before each meal and every two hours (occasionally three or four hours) throughout 24 hours. At the conclusion of the initial 24 hour study, each of the subjects was put on an isocaloric rice diet containing about 5 Gm. of fat and 20 to 30 Gm. of proteins and then on a formula diet in which corn oil (Mazola® oil [1])

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† Fellow of the Heart Association of Southeastern Pennsylvania, 1957 and 1958.

[1] Kindly supplied by the Corn Products Refining Company, Argo, Ill.

TABLE I

Summary of clinical data on subjects studied

Group	Name	Sex	Age, yrs.	Vascular disease	Serum lipid abnormality	Serum triglyceride (normal range, 25–150 mg./100 ml.)
						mg./100
I	K.B.	M	23		Normal	68–76
	J.J.	M	24		Normal	52–66
	A.P.	F	43	Pseudobulbar palsy, Lupus	Normal	91–117
	M.G.	F	46	Rheumatic mitral disease	Normal	99–138
II	R.J.	F	36	Coronary artery disease	Hypercholesteremia, "pure"	68–88
	J.Hd.	M	54	Coronary artery disease	Hypercholesteremia, "pure"	59–88
III	R.K.	M	11		Hypercholesteremia, mild hyperlipemia	321–427
	R.Ka.	M	42	Coronary artery disease	Hypercholesteremia, mild hyperlipemia	217-288
IV	J.H.	M	34	Peripheral artery disease	Essential hyperlipemia	794–945
	M.R.	M	53	Peripheral artery disease	Essential hyperlipemia	891–1,484

supplied 50 to 70 per cent of the individual's total daily caloric intake (3). This diet contained 120 to 160 Gm. of fat and 45 to 60 Gm. of proteins. During these special dietary periods, the subjects were encouraged to consume all their daily prescribed rations in order to keep their weight from varying for more than one to two pounds. Fasting blood lipid levels of these subjects were determined at weekly intervals during each dietary period. Significant changes in their serum lipids were generally observed in the second week of each dietary period. It took four to six weeks for the blood lipids of these patients to stabilize on a prescribed diet in preparation for the 24 hour test. Two subjects were kept on rice and fruit diet for 10 and 18 weeks, respectively, before their diurnal blood lipid studies were made. In three patients, the rice and corn oil formula dietary periods were repeated, and then diurnal serum triglyceride curves were again determined.

Total serum cholesterol, phospholipid, total esterified fatty acids, and triglyceride were determined in each of the blood specimens drawn. The methods of Abell, Levy, Brodie and Kendall (12), of Zilversmit and Davis (13), of Stern and Shapiro (14) and of Van Handel and Zilversmit (15) were used for the respective determinations. Serum triglyceride concentrations were obtained both by the direct determination and by calculation from cholesterol, phospholipid and total esterified fatty acid values. In each patient, the triglyceride values obtained from the 24 hour test, performed at the end of each dietary period, were utilized to plot a diurnal serum triglyceride curve. The effects of dietary fat intake upon the diurnal serum triglyceride pattern of each of these patients were compared and studied.

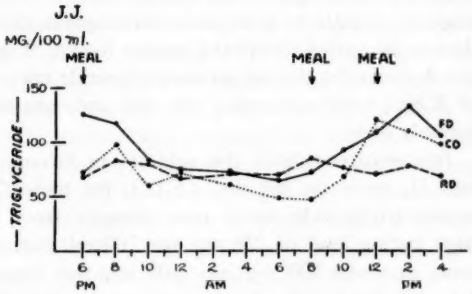

FIG. 1. DIURNAL SERUM TRIGLYCERIDE CURVES OF J.J., A 24 YEAR OLD HEALTHY, MALE, MEDICAL STUDENT

Curves F.D., C.O. and R.D. were obtained on full, corn oil formula and rice diets, respectively.

RESULTS

Diurnal serum triglyceride patterns of subjects with normal serum lipids and with "pure" hypercholesteremia (Groups I and II)

The four subjects in Group I each exhibited fairly similar diurnal serum triglyceride curves with full, rice and corn oil formula diets. The effects of dietary changes upon the diurnal serum triglyceride pattern of a young, healthy medical student (J.J.) and of a patient with rheumatic heart disease and normal serum lipids (M.G.) are shown in Figures 1 and 2, respectively. While on full diet (F.D.) the serum triglyceride curves of both subjects begin to rise sharply from their respective fasting levels soon after breakfast. These elevations were maintained throughout the day by meals with high saturated fat content. The postabsorptive lipemias then begin to decrease two to four hours after supper, and the serum triglyceride curves of both subjects are lowered to their respective fasting levels at about 12:00 p.m. When animal fats are omitted from their diet, and

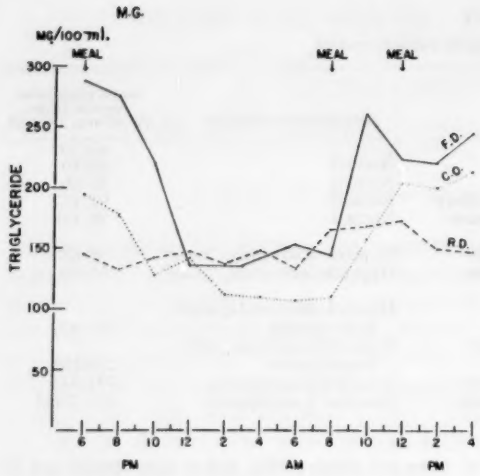

FIG. 2. DIURNAL SERUM TRIGLYCERIDE CURVES OF M.G., A 46 YEAR OLD FEMALE WITH NORMAL SERUM LIPID VALUES AND COMPENSATED RHEUMATIC MITRAL DISEASE

Curves F.D., C.O. and R.D. were obtained on full, corn oil formula and rice diets, respectively.

70 per cent of their total daily caloric intake is in the form of corn oil (C.O.), their serum triglyceride curves show more gradual rises to smaller lipemic peaks than those observed during the full diet periods. These milder postabsorptive lipemias are again maintained throughout the day. But, soon after supper, the curves drop rapidly to their respective low fasting levels (20 to 40 mg. per 100 ml. lower than the respective fasting full diet levels). On rice diet (R.D.), their serum triglyceride curves show little fluctuation with meals. After 14 hours of fasting, slight rises in their serum triglyceride concentrations are observed. The fasting baselines of these relatively flat curves run at almost exactly the same level as that of the full diet curves, and they are about 20 to 40 mg. per 100 ml. higher than those of the respective corn oil feeding periods.

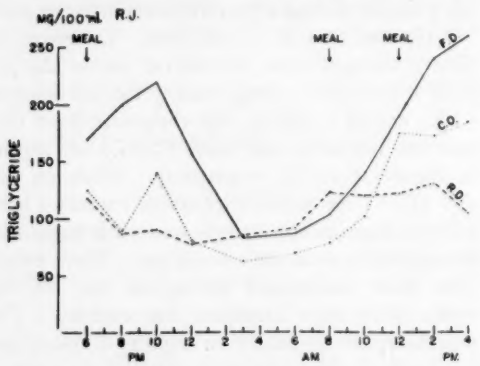

FIG. 3. DIURNAL SERUM TRIGLYCERIDE CURVES OF R.J., A YOUNG FEMALE WITH ISCHEMIC HEART DISEASE AND "PURE" HYPERCHOLESTEREMIA

Curves F.D., C.O. and R.D. were obtained on full, corn oil formula and rice diet, respectively.

The patients with "pure" hypercholesteremia and low serum triglyceride concentration show essentially the same diurnal triglyceride patterns in response to these dietary manipulations. The diurnal serum triglyceride curves obtained from R.J. with rice diet and high saturated and unsaturated fat intakes are shown in Figure 3.

The diurnal serum triglyceride level studies with rice and corn oil formula diets were repeated in M.G. and R.J. three to four months after the completion of the initial series of determinations. Excellent reproductions of the original rice and corn oil serum triglyceride patterns were obtained in each of the two patients. It is of interest to note that both the fasting and the postprandial serum triglyceride concentrations of the two young medical students were lower than those observed in the four older patients having either normal serum lipid concentrations or "pure" hypercholesteremia.

Diurnal serum triglyceride pattern of patients with hypercholesteremia and mild hyperlipemia

Two patients with hypercholesteremia and mild degrees of hyperlipemia had similar diurnal serum triglyceride patterns in response to changes in their dietary fat, carbohydrate and protein intake. Figure 4 shows the diurnal serum triglyceride curves of R.Ka., while consuming full, rice and corn oil formula diets.

In comparison with the subjects in Group I and II, while on full diet (F.D.), the patient's serum triglyceride curve rose abruptly from a high fasting level of 225 mg. per 100 ml. fasting level to about 500 mg. per 100 ml. two hours later. Subsequently, the curve shows two lipemic peaks, one following lunch and the other after supper. The postabsorptive lipemia is prolonged and is "cleared" by 4:00 a.m. The curve then remains at this relatively low level until the next

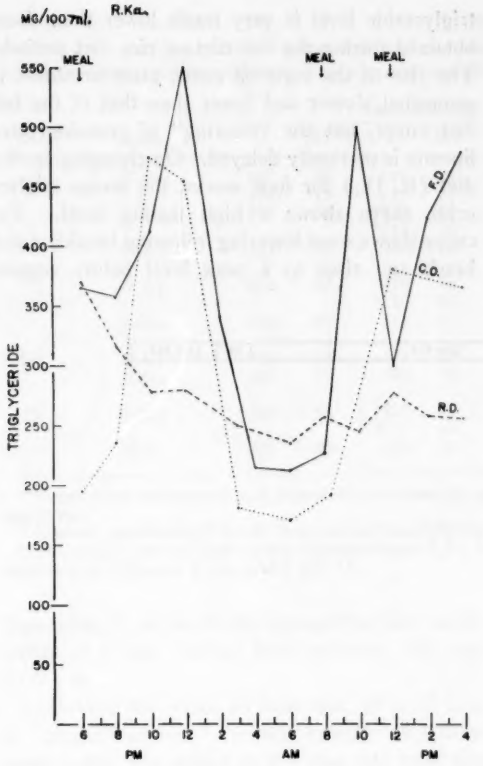

FIG. 4. DIURNAL SERUM TRIGLYCERIDE CURVES OF R.KA. ON FULL (F.D.), CORN OIL FORMULA (C.O.) AND RICE (R.D.) DIETS

Patient had ischemic heart disease and moderate elevation of serum cholesterol and triglyceride levels.

breakfast. Like the subjects in Group I and II, with corn oil feeding (C.O.), the rise in the serum triglyceride curve following breakfast is more gradual and reaches a lower peak than that of the postabsorptive curve obtained on him while he consumed a full diet. However, the lipemia is quite marked and prolonged in the early evening. The curve dips to a low level (30 to 40 mg. per 100 ml. lower than the fasting values of the full diet curve) in the early morning. It is then maintained at 170 to 180 mg. per 100 ml. level until the following meal. The rice diet curve (R.D.) shows no marked postabsorptive fluctuations. It is interesting to point out that, while the patient was on rice diet, the serum triglyceride levels measured during the fasting hours were considerably higher than those taken when the patient was either on a full diet or on a high corn oil intake. In spite of the very low fat content of the rice and fruit diet, the serum triglyceride curve shows a moderate elevation in the early evening.

Diurnal serum triglyceride of hyperlipemic subjects

Alterations in the diurnal serum triglycerides with dietary changes in the two patients with es-

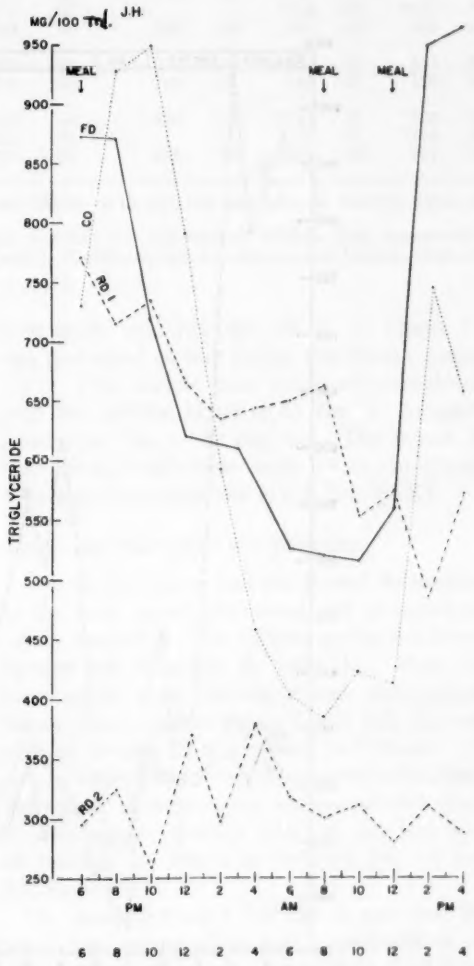

FIG. 5. DIURNAL SERUM TRIGLYCERIDE CURVES OF J.H., A HYPERLIPEMIC WITH PERIPHERAL ARTERIAL OCCLUSIVE DISEASE

Curves F.D., C.O. and $R.D._1$ and $R.D._2$ were obtained on full, corn oil and rice (Periods 1 and 2) diets, respectively.

sential hyperlipemia were quite similar. Figure 5 shows the studies obtained from J.H. The full diet curve (F.D.) shows that his serum triglyceride rises sharply from a highly elevated fasting level following breakfast to reach a very high level at 12:00 noon. This high serum triglyceride concentration is maintained by the subsequent meals. The curve then returns to a high baseline at 6:00 a.m. and remains unchanged before the next breakfast. While on corn oil (C.O.), his fasting serum triglyceride level is very much lower than those obtained during the full diet or rice diet periods. The rise of the corn oil curve after breakfast is somewhat slower and lower than that of the full diet curve, but the "clearing" of postabsorptive lipemia is markedly delayed. On changing to rice diet (R. $D._1$) for four weeks, the serum triglyceride curve shows a high fasting level. The curve shows some lowering following breakfast and lunch and rises to a peak level before supper.

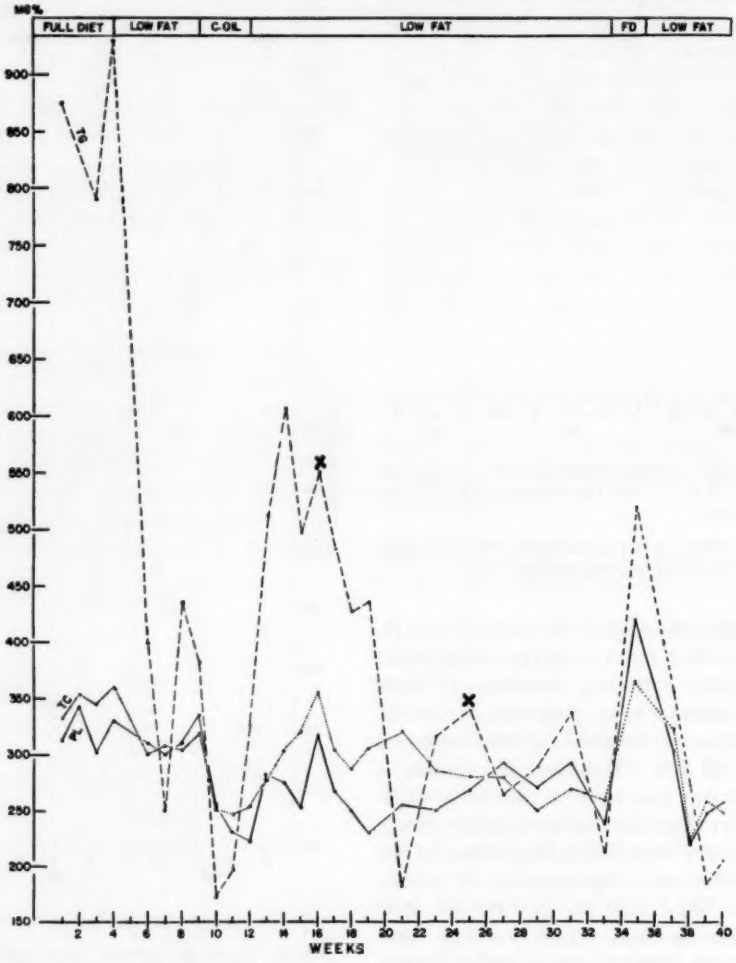

FIG. 6. CHANGING SERUM TRIGLYCERIDE (T.G.), TOTAL CHOLESTEROL (T.C.) AND PHOSPHOLIPID (P.L.) LEVELS OF J.H. WITH DIETARY CHANGES

F.D. indicates full diet. X and XX indicate the times at which the diurnal triglyceride curves R.$D._1$ and R.$D._2$ of Figure 5 were obtained.

TABLE II

Effect of diet on serum cholesterol and phospholipid concentrations of the subjects studied

Group	Name	Total cholesterol						Phospholipids					
		1*	1A†	2*	2A†	3*	3A†	1	1A	2	2A	3	3A
I	K.B.	167	10	119	16	117	15	182	30	135	18	123	27
	J.J.	144	19	113	17	109	17	181	32	136	20	132	20
	A.P.‡	275	34	[a]222	23	[a]197	31	203	43	[a]199	30	[a]183	53
				[b]212	30	[b]204	25			[b]189	32	[b]175	38
	M.G.	257	32	223	31	198	34	310	95	253	35	219	55
II	R.J.‡	435	67	[a]319	48	[a]254	59	303	83	[a]209	40	[a]178	73
				[b]326	45	[b]270	57			[b]218	57	[b]183	59
	J.Hd.	470	59	361	44	308	40	285	68	232	62	206	67
III	R.K.	987	104	673	80	891	108	658	104	407	28	512	80
	R.Ka.	325	55	253	30	250	40	249	65	242	67	238	44
IV	J.H.‡	383	93	[a]321	42	[a]245	44	420	118	[a]297	50	[a]248	78
				[b]271	40	[b]233	31			[b]220	35	[b]254	59
	M.R.	342	63	259	39	239	54	458	80	251	30	211	41

* Serum total cholesterol and phospholipid values in mg. per 100 ml. with full, rice and corn oil formula diets, respectively.

† Diurnal varixtions of levels (maximal-minimal differential) with full, rice and corn oil formula diets, respectively.

‡ Two sets of serum lipid values obtained from A.P., R.J. and J. H.while on rice diet and corn oil formula diets are presented in Columns 2, 2A, and 3 and 3A.

Thereafter, it drifts slowly downwards and finally settles at a high fasting level between 2:00 and 8:00 a.m.

Following the initial 24 hour test, 60 to 65 Gm. of Amigen® powder [2] (protein hydrolysate) three times a day was added to the rice and fruit diet of this patient. A second 24 hour serum triglyceride study was performed on him six weeks later. The results are presented graphically ($R.D._2$). Except for two minor elevations at 12:00 midnight and 4:00 a.m., respectively, the curve is quite flat and low.

The effect of dietary changes upon the fasting serum lipid levels of this hyperlipemic patient, J.H., is shown in Figure 6. While on full diet with high saturated fat intake, his fasting serum triglyceride concentration ranged between 794 to 945 mg. per 100 ml. A precipitous drop in his serum lipid concentration, particularly that in his serum triglyceride level, was observed when he was placed on rice (low fat) diet. The subsequent period of corn oil feeding apparently caused a further lowering of his serum lipid values. However, on returning to the rice (low fat) diet, the patient developed a neutral-fat lipemia which lasted for 10 weeks. The first 24 hour blood lipid study with rice diet ($R.D._1$ in Figure 5) was performed on him during this lipemic period (X). This lipemic state improved coincidental with the addition of 60 to 65 Gm. of Amigen® powder per day to the rice diet. The second 24 hour serum triglyceride study ($R.D._2$ in Figure 4) was performed on him at this time (XX).

[2] Kindly supplied by Dr. E. A. Hawk, Mead Johnson Company, Evansville, Ind.

Serum cholesterol and phospholipids

The 24 hour mean and the diurnal fluctuations in the total serum cholesterol and phospholipid values obtained in these patients during this investigation are tabulated in Table II. Mean 24 hour serum total cholesterol and phospholipid values of each patient obtained with full, rice and corn oil formula diets are listed in Columns 1, 2 and 3, respectively. The diurnal maximal-minimal differential of their serum cholesterol and phospholipid values observed with full, rice and corn oil feedings are shown in Columns 1A, 2A and 3A, respectively.

On changing from a full diet to rice diet, the mean total serum cholesterol concentrations of these subjects showed an average drop of 23.3 per cent (range, 13.2 to 31.8 per cent). And, their mean serum cholesterol levels observed during the periods of corn oil formula diet were on the average 27.0 per cent (with a range of 9.7 to

36.0 per cent) lower than those obtained during the full diet periods. Somewhat similar lowerings of their mean serum phospholipid values were also observed. With the changes in the diet, their mean serum phospholipid levels were 26.5 per cent (range, 1.8 to 47.0 per cent) and 29.1 per cent (range, 4.4 to 53.8 per cent) with rice and corn oil formula diets, respectively, lower than values observed on them during the full diet periods.

A high corn oil intake appears to be more effective than rice diet in lowering the serum cholesterol and phospholipids level in five subjects. However, the degree of effectiveness of corn oil in lowering the serum lipids varied a great deal from one subject to another. In three subjects, the serum cholesterol and phospholipid concentrations were not significantly lowered on changing from rice and fruit diet to corn oil formula feeding.

In the case of R.K., his serum phospholipid and cholesterol increased more than 100 mg. per 100 ml. with corn oil feeding for 12 weeks.

Like the serum triglyceride concentrations, the serum cholesterol and phospholipid levels of these subjects also showed a tendency to diurnal variations. In these subjects, the lowest points of their lipid curves were usually inscribed between the hours of 4 and 8 a.m., and the highest levels were registered between 2 to 9 p.m. The 24 hour maximal-minimal differential of their serum cholesterol and phospholipids concentrations ranged between 15 to 108 mg. per 100 ml. and 18 to 118 mg. per 100 ml., respectively, in these patients. Diurnal fluctuations of these serum lipid levels were particularly prominent in patients with extreme degrees of hypercholesterolemia and hyperphosphatemia, while ingesting a full diet with a high saturated fat intake. The lipid curves tended to flatten out after the serum lipid concentrations of these subjects were lowered by the use of rice and corn oil formula diets.

DISCUSSION

Much interest has recently been aroused in the study of serum triglyceride concentrations by the demonstration that hyperlipemia may accelerate blood clotting (16–20), increase blood viscosity as well as the adhesiveness and aggregation of red blood cells (21–23), and aggravate myocardial ischemia (24). An increased coagulability of the blood was also observed in patients with ischemic heart disease, ingesting a full diet. The platelet stickiness was found to decrease with the use of rice-fruit diet (25, 26). Recently, Albrink and Man (4) have reported a better correlation between fasting serum triglyceride concentration and the incidence of clinical atherosclerosis than that of serum cholesterol levels. It has been shown that both fat and carbohydrate ingestion can cause a rapid and profound change in the concentration of this labile serum lipid fraction (5–11). Consequently, adequate information of the dietary effect upon the serum triglyceride level in a given patient can only be obtained by studying the changing concentrations of the lipid in his blood throughout the 24 hour period.

In the attempt to elicit evidence of a defective lipid metabolism in human atherosclerosis, a number of fat tolerance tests have been employed by various investigators to study the nature of postabsorptive lipemia in atherosclerotic subjects. Although certain abnormalities in the postprandial lipemia pattern have been observed in the majority of patients with clinical atherosclerosis, however, the data obtained were beclouded by a number of uncontrolled factors. It must be pointed out that, in addition to the individual's fat tolerance, his previous dietary habit (10), the kind and the amount of fat given, as well as glucose, sugars and carbohydrate used in the test meal (11, 27–30) may profoundly affect his postprandial serum triglyceride curve. In the present study a diurnal serum triglyceride curve was obtained in each of the subjects after he had been stabilized in a given dietary regimen. This experimental design would tend to eliminate most of the complicating factors mentioned; and it would permit a comparative study of: *a*) differences in fat tolerance between one individual and another, *b*) differences in postabsorptive lipemias caused by ingestion of large amounts of saturated (typical American diet) and unsaturated fat [corn oil formula diet as described by Ahrens and coworkers (3)].

The data obtained from the study of this small series of subjects indicate that while on corn oil formula diet, the postprandial serum triglyceride elevation in each of the subjects, with normal serum lipid concentration and with "pure" hypercho-

lesteremia, was significantly lower and briefer than the elevation obtained while he was on a full diet. These differences in the effect of saturated and unsaturated dietary fat upon the serum triglyceride became progressively less evident in patients with increasing degrees of hyperlipemia. It is well known that the measured serum triglyceride level represents the resultant of triglyceride absorption into, and of its removal from, one's blood stream. Since there is no apparent evidence for a disturbed fat absorption from the gastrointestinal tract of any of these patients, it is perhaps reasonable to assume that in certain subjects, particularly in the hypercholesteremics, the rate of triglyceride removal from the blood stream is probably increased when they are consuming a diet with high unsaturated fat content.

Of further interest is the observation of a progressively rising serum triglyceride level before supper in hyperlipemic patients, while consuming a rice and fruit diet, which contains only about 5 Gm. of fat per day. The mechanism of this predinner rise of serum triglyceride concentration is not well understood at the present time. Since the body store of carbohydrate is limited, triglycerides (its lability may be increased in patients with hyperlipemia) may have been mobilized from the fat depot to satisfy the energy requirements of the patient when the availability of carbohydrate for energy metabolism is decreased. Havel, in his study of effects of glucose administration on very low density lipoprotein of clinically normal men, has suggested that its concentration appears to depend on the availability of carbohydrate for energy production (10).

The chief interest of this investigation is centered on the observation that certain patients would exhibit a lipemia with severe restriction of daily dietary fat intake (1–4). On this basis, Ahrens and his associates (3) have repeatedly cautioned against the use of a low fat diet in patients with ischemic heart disease. It should be emphasized, however, that all of their observations were confined to blood lipid determinations made in the fasting state. Our data showed that in a nonhyperlipemic subject, the fasting serum triglyceride level was not significantly affected by extreme changes made in the dietary fat content. But in a hyperlipemic patient, the fasting serum triglyceride level may show a considerable elevation with the prolonged use of rice diet thus confirming the observations made by Hatch, Abell and Kendall (2), Ahrens and associates (3) and Albrink and Man (4). However, if this comparative study of serum triglyceride concentration with changing dietary fat content were extended to include the whole postabsorptive period (8:00 to 2:00 a.m.) of the day, it would be difficult to demonstrate that the addition of large quantities of corn oil to the diet had actually lowered the diurnal serum triglyceride levels of the patient. Recently, McDonald and Edgill have demonstrated a decrease in platelet adhesiveness in patients with ischemic heart disease while consuming a rice and fruit diet (26). Although no attempt has been made by these investigators to correlate their findings with serum triglyceride concentrations of their patients, it is nevertheless valid to assume that some of their patients must have developed lipemia with the rice diet; and that this fasting lipemia of fat restriction type may exert totally different physiologic effects from that of postabsorptive type of lipemia following the ingestion of a high fat meal.

The mechanism of the fasting lipemia with rice diet is not entirely understood. Hatch, Abell and Kendall (2) suggested that the phenomenon may be due to the conversion of carbohydrate into fat when food intake exceeds energy requirements. The possibility of other metabolic factors including an increased triglyceride mobilization also being involved in the production of this peculiar type of lipemia remains to be elucidated.

In all subjects the serum triglyceride concentrations obtained by calculation from cholesterol, phospholipid and total esterified fatty acid values were consistently lower than those obtained by the direct method. The amount of difference observed between the two methods varies considerably from one subject to another. Negative values were obtained by the calculation method in both hypercholesteremic patients (R.J. and J.Hd.). Hence, only the values obtained by the direct method were reported. Entenman (31) suggested and Reinhold (32) recently demonstrated that this underestimation of triglycerides by calculation may be due to the fact that various cholesterol esters yield low molar estimation values. Moreover, a portion of phospholipid is present in plasma as sphingomyelin which con-

tains only one fatty acid molecule per mole of phosphorus.

SUMMARY

1. The diurnal variations in serum triglyceride, cholesterol and phospholipid levels of a series of 10 subjects on isocaloric full, rice and fruit and 50 to 70 per cent corn oil formula diets were determined.

2. In all subjects the diurnal serum cholesterol and phospholipid variations were minimal.

3. In four subjects with normal lipid metabolism and two patients with "pure" hypercholesteremia the postprandial serum triglyceride elevations were lower and briefer while on corn oil diet than on a typical American (full) diet.

4. These differences in the effects of unsaturated and saturated dietary fats upon one's postprandial serum triglyceride concentration became progressively less evident in four patients with increasingly severe degrees of hyperlipemia.

5. While on rice and fruit diet, the fasting serum triglycerides of the nonlipemic subjects were only slightly higher than their respective fasting levels measured during the full and corn oil formula dietary periods. But the mean diurnal serum triglyceride levels of these subjects were low with the rice and fruit diet.

6. In hyperlipemic patients, significant inincreases in their fasting serum triglyceride concentrations were observed, following the change from high fat diets to the rice and fruit diet.

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THE EFFECT OF 6-MERCAPTOPURINE ON PRIMARY AND SECONDARY IMMUNE RESPONSES *

By ROBERT SCHWARTZ,† ANNA EISNER AND WILLIAM DAMESHEK

(From the Blood Research Laboratory, New England Center Hospital, and the Department of Medicine, Tufts University School of Medicine, Boston, Mass.)

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The immune response may be considered as a complex metabolic process initiated by an antigenic substance and resulting in the formation of highly specific proteins. The mechanisms underlying this response are not clearly understood, but it is evident that the nucleus of antibody forming cells as well as their cytoplasm participate (1). Following the administration of antigenic material to experimental animals, striking morphologic and biochemical changes occur within the antibody forming tissues (2). The structural alterations include hypertrophy and cellular proliferation, while the biochemical changes are reflected by increased nitrogen, desoxyribose nucleic acid (DNA) and ribonucleic acid (RNA) contents of these tissues. These changes occur during the primary and secondary immune responses [1] as well as after the stimulation of regional lymph nodes by heterografts (3). The increase in DNA content is largely due to cell multiplication, which begins within 48 hours after the injection of antigen. Increases in RNA, however, occur somewhat later, and the peak of cellular RNA concentration coincides closely with the peak of serum antibody titer (4, 5). Studies such as these indicate that the formation of antibody is closely linked to nucleic acid metabolism.

In a previous publication we have shown that a purine analog, 6-mercaptopurine (6-MP), prevents the formation of antibodies by hyperimmunized rabbits (6). In this report we will present further studies concerning the mechanism of action of 6-MP on the immune responses of rabbits. These responses were studied *in vivo* by determining rates of antigen disappearance using the isotope technique of Talmage, Dixon, Bukantz and Dammin (7) and by measuring antibody appearance by the ammonium sulfate fractionation method of Farr (8) and the tannic acid hemagglutination of Stavitsky (9). It will be shown that 6-MP can completely block the primary immune response, while that dose of the drug which prevents the appearance of the primary response delays, but does not prevent, the onset of the secondary response.

MATERIALS AND METHODS

Animals. Healthy white New Zealand rabbits obtained from a nearby supplier and weighing about 2 Kg. each were used. The animals were housed in individual cages in an air-conditioned room. All rabbits were maintained on Purina Rabbit Chow® and water *ad libitum*.

Radioisotopes and antigens. Radioiodinated human serum albumin (HSA-I) was obtained from Abbott Laboratories. This material is supplied in vials containing about 1 ml. of a 1 per cent solution of human serum albumin conjugated with 500 μc. of I-131. "Loosely bound" radioiodine activity of this preparation is less than 1 per cent, and the appearance of free iodide during storage is negligible. Human serum albumin (HSA) was obtained from the Protein Foundation Laboratories (Lot No. HAB51CH). This material was isolated from human serum by the cold ethanol fractionation method of Cohn, and was supplied to us as a dry powder. Solutions of the desired concentration were prepared in 0.15 M saline.

Isotope detection. Isotope activity was detected in a well-type scintillation counter which affords reproducible counts better than $\pm$ 3 per cent. The detector was standardized daily against a long-lived nuclide, Cs 137. I-131 counts were made directly from measured amounts of serum, usually 1.0 ml. In order to eliminate counting

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[1] The primary response is an immunological reaction which follows the first exposure to an antigen. It is characterized by the relatively slow appearance of small quantities of antibody in the blood. The secondary response is an immunological reaction which occurs after re-exposure to an antigen. In contrast to the primary response, this reaction is rapid, large quantities of antibody are formed and antibodies are found in the blood over a long period of time.

errors due to geometric variations of the sample, those tubes which contained less than this amount of serum were diluted to 1.0 ml. with 0.15 M saline. Serum I-131 activity of the various samples was corrected for individual variations in blood volume, assuming a blood volume of 8 ml. per Kg. of body weight. Of those sera which contained radioactivity, no sample had counts less than twice that of the background radioactivity.

6-Mercaptopurine. This compound was supplied as an amorphous yellow powder, insoluble in water, but quite soluble in dilute alkali. Fresh solutions were prepared daily by dissolving pre-weighed aliquots of 6-MP in 1.0 N NaOH (1 ml. of alkali per 100 mg. of 6-MP); the resulting solution was diluted to the desired concentration with normal saline. All injections of the drug were given intramuscularly.

TABLE I

*Effect of 6-mercaptopurine on the primary immune response**

	Day after antigen injection										
No.	1	3	5	7	8	9	10	12	14	15	16
Controls											
1	35.6	21.2	16.1	5.0	2.1	0.3	0.0				
2	34.2	22.1	14.2	7.2	3.4	0.4	0.0				
3	31.0	21.4	11.3	6.3	2.7	0.6	0.0				
4	33.7	23.2	14.0	6.4	5.4	1.6	0.0				
5	33.2	24.3	14.1	8.6	3.4	0.6	0.0				
6	31.4	19.5	9.5	6.1	3.2	0.3	0.0				
35	32.5	17.3	12.7	8.3	3.9	1.0	0.0				
36	38.7	22.8	14.1	10.7	6.6	1.2	0.0				
37	36.9	26.9	17.1	13.8	8.0	1.8	0.0				
38	42.0	26.8	15.5	13.4	6.9	0.9	0.0				
39	41.1	25.5	15.1	8.7	5.1	0.8	0.0				
Avg.	39.0	25.1	15.4	9.5	5.1	1.0	0.0				
1 mg./Kg./day											
66	37.0			13.1	10.2	8.1	6.4	4.5	2.4		
67	40.3			15.0	10.5	7.4	5.0	0.8	0.0		
68	41.1			15.5	11.4	8.4	5.1	0.7	0.0		
69	35.5			9.7	6.1	4.1	3.7	0.9	0.0		
70	46.0			15.6	11.7	8.8	5.7	1.9	0.0		
Avg.	40.0			13.8	10.0	7.4	5.2	1.8	0.5		
2 mg./Kg./day											
55	31.6	23.2	16.7		10.8	8.3	7.1	4.5	0.2	0.0	
56	44.3	26.7	10.1		9.3	7.2	5.2	3.2	0.9	0.0	
57	49.5		19.8		11.5	8.6	8.4	4.7	0.9	0.0	
58	43.4	28.4	15.9		8.3	9.8	4.1	5.3	0.8	0.0	
59	45.9	35.7	19.7		8.6	7.4	6.2	3.6	0.6	0.0	
Avg.	42.9	28.5	16.4		9.7	8.3	6.2	4.3	0.6	0.0	
3 mg./Kg./day											
7	34.7	21.5	12.6	8.7	7.8	6.5		4.0	2.1	0.2	0.0
8	32.1	23.5	16.8	12.0	10.6	9.7		7.7	4.2	0.1	0.0
9	33.6	26.0	18.5	16.0	13.8	13.6		12.6	5.8	0.8	0.0
10	34.0	24.1	15.0	12.7	11.6	9.8		8.0	3.7	0.7	0.0
22	34.1	19.7	15.5	10.8	8.3	7.2		6.3	3.6	0.4	0.0
Avg.	33.7	21.2	15.7	12.0	10.4	9.4		7.7	3.9	0.4	0.0
6 mg./Kg./day											
152	29.3			10.1			6.4	5.4	2.7		1.9
153	36.2			8.2			5.5	5.1	2.1		1.5
154	33.9			7.1			4.8	3.8	1.6		0.9
155	29.0			3.7			4.0	2.7	1.1		0.7
156	40.8			8.4			5.2				
157	26.1			8.2			5.5	4.7	2.0		
161	24.2			11.1			5.5		3.4		2.4
162	31.5			14.1			8.2		5.5		3.5
163	30.4			16.0			8.6		5.8		2.6
164	37.3			15.3			8.0		6.0		3.2
165	29.2			13.5			6.6		1.7		0.5
166	30.7			14.2			12.0		7.9		4.3
Avg.	31.6			10.8			6.7	4.3	3.4		1.9

* The value for each animal represents percentage of initial radioactivity.

TABLE II

Percentage of globulin-bound antigen appearing during the primary response

No.	Day after antigen injection									
	1	3	5	7	8	9	10	12	14	16
Controls										
1	2.1	1.9	1.7	2.3	18.3	88.3	0.0			
2	1.2	3.2	2.8	1.6	54.0	93.2	0.0			
3	1.4	2.1	2.1	1.6	41.2	78.6	0.0			
4	1.7	1.6	3.7	4.3	36.8	84.3	0.0			
5	0.8	1.9	2.3	0.7	58.3	98.0	0.0			
6	3.6		0.7	1.2	75.3	67.8	0.0			
35	1.2	1.4	0.8	1.4	48.3	87.2	0.0			
36	4.1	6.1	2.3	11.0	62.3	100.0	0.0			
37	2.5			17.0	51.4	93.0	0.0			
38	0.6	2.6	2.8	6.3	46.9	87.6	0.0			
39	1.4	3.8	1.1	0.7	79.8	96.3	0.0			
Avg.	1.9	2.7	2.0	4.8	45.9	97.4	0.0			
6-MP, 6 mg./Kg./day										
152	1.6			4.6			2.2	0.3	1.6	1.5
153	3.2			1.4			1.6	2.3	2.4	1.7
154	0.9			1.9			0.9	2.3	1.4	1.9
155	1.7			4.1			3.4	3.1	1.4	2.9
156	1.5			1.3			3.2	2.4	1.5	3.2
157	1.3			7.5			1.4	1.1	0.8	1.7
161	1.2			3.5			2.1	1.0	2.4	1.8
162	3.4			2.4			3.2	1.6	7.1	1.0
163	1.4			3.5			0.8	1.2	3.4	1.9
164	0.7			2.3			0.7	1.6	1.2	3.1
165	1.1			4.3			1.9	2.0	1.0	0.9
166	0.9			3.2			1.1	1.0	5.4	3.2
Avg.	1.6			3.3			1.8	1.7	2.5	2.1

Estimation of antibody production. Two methods were used: *1*) After the radioactivity of the serum samples had been determined, sufficient saturated ammonium sulfate was added to produce a final concentration of 50 per cent ammonium sulfate. The precipitated globulins were washed three to four times in half-saturated ammonium sulfate, or until the supernatant contained no radioactivity. After discarding the supernatant, the radioactivity of the precipitate was measured as described above. The radioactivity of the washed globulin precipitate indicated the relative concentration of circulating antigen–antibody complexes since only the human albumin firmly bound to rabbit globulin precipitated in the half-saturated ammonium sulfate. *2*) "Free" serum antibody was measured at various intervals by the tannic acid hemagglutination method of Stavitsky (9), with one minor modification, namely, normal saline was substituted for buffered (pH 7.4) saline, since in our hands this change produced sharper agglutination patterns. Results of these tests were expressed as the $\log_2$ of the highest dilution of serum showing a 1+ pattern.

Immunization, dosage and bleeding schedules. 1) Primary response: Thirty-eight animals, 11 in the control group and 27 in the experimental groups, were used. Each animal was given one intravenous injection of 10 to 15 μc. of HSA-I mixed with 20 mg. of "carrier" HSA. The experimental groups were given 6-MP in the following dosage schedules: 1 mg. per Kg. per day, 2 mg. per Kg. per day, 3 mg. per Kg. per day and 6 mg. per Kg. per day. Administration of the drug was begun on the first day of antigen injection (Day "0") and was continued for 13 days. Blood was obtained from the marginal ear veins. An initial bleeding was obtained 15 minutes after the injection of the antigen and the counts per minute of this sample were taken as 100 per cent radioactivity. Bleedings were made either daily or every second or third day, as indicated in the Results.

2) Secondary response. Eighteen animals, eight in the control group and 10 in the drug treated groups, were used. Each animal was given a single intravenous injection of 100 mg. of HSA. Six to 12 weeks later a second injection of 10 to 15 μc. of HSA-I together with 20 mg. of "carrier" protein were administered intravenously. The experimental groups were given 6-MP in the following doses: 3 mg. per Kg. per day for five days and 6 mg. per Kg. per day for eight days, beginning with the second injection of antigen. Blood was obtained as for the primary response.

RESULTS

The primary response

The results of these experiments are illustrated in Figures 1, 2 and 3 and the data for each rabbit are listed in Tables I, II and III. The disappearance of HSA-I from the serums of the untreated animals occurred in three distinct phases,

TABLE III

*Production of humoral antibody during the primary response**

No.	Day after antigen injection					
	1	5	9	12	18	22
Controls						
1	0	0	0	7.3	10.4	8.3
2	0	0	0	6.3	11.4	7.3
3	0	0	0	6.3	11.4	7.3
4	0	0	0	3.3	11.4	10.4
5	0	0	0	5.3	9.3	10.4
35	0	0	0	6.3	10.4	7.3
36	0	0	0	7.3	10.4	8.3
37	0	0	0	8.3	11.4	8.3
38	0	0	0	11.4	14.4	10.4
39	0	0	0	6.7	8.3	7.3
Avg.	0	0	0	6.7	10.9	8.5
6-MP, 6 mg./Kg/day						
152	0	0	0	0	0	0
153	0	0	0	0	0	0
154	0	0	0	0	0	0
155	0	0	0	0	0	0
156	0	0	0	0	0	0
157	0	0	0	0	0	0
161	0	0	0	0	0	0
162	0	0	0	0	0	0
162	0		0	0	0	0
163	0		0	0	0	0
164	0		0	0	0	0
165	0		0	0	0	0
166	0		0	0	0	0
Avg.	0	0	0	0	0	0

* Titers for tannic acid hemagglutination tests; values are expressed as the $\log_2$ of the highest dilution giving a 1+pattern.

corresponding closely to the results obtained by Talmage and associates (7), and by Dixon (10). During the first 24 hours about two-thirds of the initial radioactivity disappeared from the serum. The remaining one-third then showed a logarithmic decay curve similar to that seen with other circulating proteins. However, on the seventh day after antigen injection an accelerated disappearance phase began, and within three days all traces of radioactivity were gone from the circulation. Coincident with this accelerated disappearance of antigen ("immune disappearance phase") there was a sharp rise in the percentage of HSA-I bound to globulin, indicating the appearance of circulating antigen-antibody complexes. Nine days after antigen injection, practically all of the detectable isotope was in the globulin fraction. Twelve days after the administration of the antigen the tannic acid hemagglutination tests were positive, indicating the presence of "free" humoral antibody. Antibody titers reached their peaks about 18 days after the injection of antigen and thereafter began to decline.

All groups of animals treated with 6-MP behaved quantitatively differently from the control group, except the group treated with 6 mg. per Kg. per day, which differed both quantitatively and qualitatively from the untreated animals. This latter group will be discussed separately. The immune disappearance phase developed in each of the other groups of treated animals, but the time of onset was delayed in proportion to the dose of 6-MP administered. Thus, in the group given 1 mg. per Kg. per day the accelerated portion of the curve began on the ninth day; those animals treated with 2 mg. per Kg. per day developed this phase on the twelfth day; while the group in which 3 mg. per Kg. per day was given responded on the fourteenth day. This relationship between dose and suppression of the immune response was arbitrarily expressed as a percentage

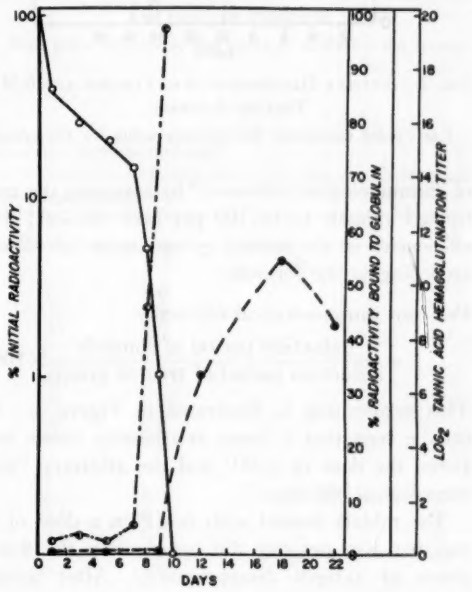

FIG. 1. ANTIGEN DISAPPEARANCE AND ANTIBODY APPEARANCE FOLLOWING THE INTRAVENOUS ADMINISTRATION OF HSA-I TO CONTROL ANIMALS

O—O—O, per cent initial radioactivity remaining in serum; O- - -O- - -O, per cent radioactivity bound to globulin; O— —O— —O, tannic acid hemagglutination titer. Each point represents the average value for the group.

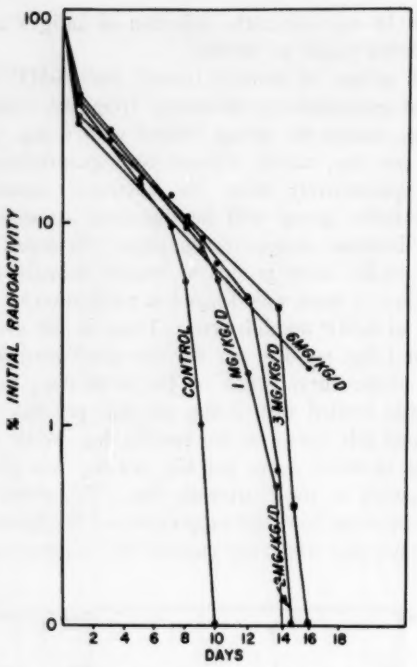

FIG. 2. ANTIGEN DISAPPEARANCE IN CONTROL AND 6-MP TREATED ANIMALS

Each point represents the average value for the group.

of "immunological efficiency" by assuming the untreated animals to be 100 per cent efficient; the efficiencies of the treated groups were calculated according to the formula:

$$\text{Per cent immunological efficiency} = \frac{\text{induction period of controls}}{\text{induction period of treated group}} \times 100.$$

This relationship is illustrated in Figure 4. It can be seen that a linear relationship exists between the dose of 6-MP and the arbitrary "immunological efficiency."

The rabbits treated with 6-MP in a dose of 6 mg. per Kg. per day did not develop the third phase of antigen disappearance. After initial equilibration, the antigen decayed logarithmically until the sixteenth day after its injection; further determinations of serum radioactivity were not done because the very small amount of isotope remaining precluded accurate count. At no time did antigen-antibody complexes appear. Humoral antibodies were not detected during the 25 days of the study. Further evidence that antibody production was completely blocked was obtained by reinjecting this group with a second dose of HSA-I. These results, which will be reported in detail elsewhere (11), indicate that antibody was absent from the blood of these animals at the time of the second injection of antigen.

The secondary response.

The results of these experiments are illustrated in Figures 5 and 6 and the data for each rabbit are listed in Tables IV, V and VI. Radiolabeled antigen disappeared from the circulation of the control group in the three distinct phases previously described. The onset of the immune disappearance phase, however, occurred much earlier —on the third day after antigen injection, and by the fifth day no circulating antigen was detectable. On the fourth day most of the circulating antigen was bound to globulin as antigen-antibody complexes. Five days after the injection of antigen "free" humoral antibody was detected by the tannic acid hemagglutination method; titers

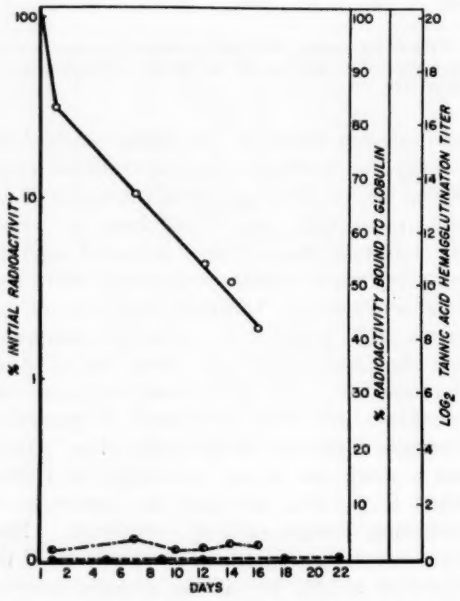

FIG. 3. ANTIGEN DISAPPEARANCE AND LACK OF ANTIBODY FORMATION IN ANIMALS TREATED WITH 6-MP (6 MG. PER KG. PER DAY) FOR 13 DAYS

Each point represents the average value for the group.

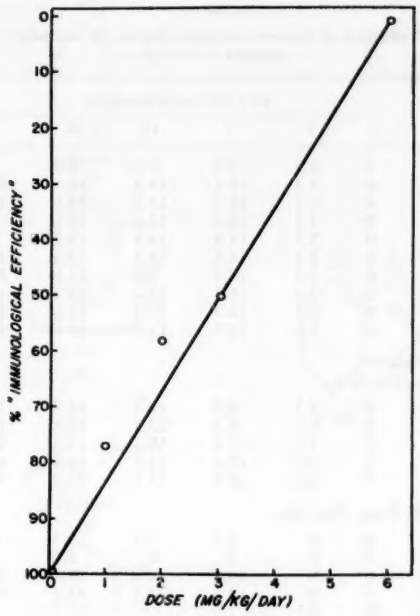

FIG. 4. RELATIONSHIP BETWEEN DOSE OF 6-MP AND DEPRESSION OF "IMMUNOLOGICAL EFFICIENCY"

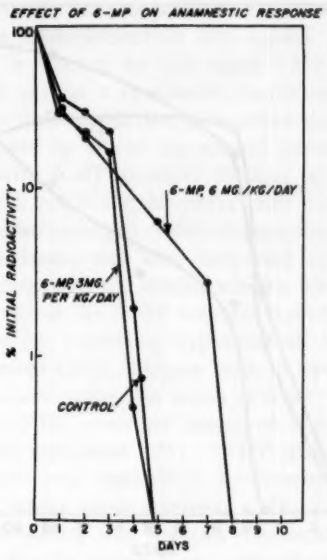

FIG. 5. ANTIGEN DISAPPEARANCE DURING THE SECONDARY IMMUNE RESPONSE

Each point represents the average value for the group.

TABLE IV

*Effect of 6-mercaptopurine on the secondary immune response**

No.	Day after antigen injection								
	1	2	3	4	5	6	7	8	9
Controls									
1	44.4	32.8	17.2	2.1	1.3	0.0			
2	39.0	29.9	16.3	0.7	0.2	0.0			
3	36.6	30.6	15.4	4.5	0.7	0.0			
5	39.9	36.0	18.2	0.9	0.1	0.0			
55	37.4		27.5	2.8	0.1	0.0			
57	38.6		28.5	1.7	0.0	0.0			
58	40.0		36.6	1.8	0.1	0.0			
59	49.2		34.4	0.1	0.0	0.0			
Avg.	40.6	32.3	24.2	1.8	0.3	0.0			
6-MP, 3 mg./Kg./day									
29	40.5	32.8	18.3	0.9	0.2	0.0			
30	35.3	20.6	17.6	1.8	0.3	0.0			
31	29.1	15.5	10.4	0.1	0.1	0.0			
32	30.5	20.0	11.3	0.7	0.0	0.0			
Avg.	33.9	22.3	14.4	0.9	0.2	0.0			
6-MP, 6 mg./Kg./day									
116	30.3	25.9	18.8	13.6	9.0		3.8	0.1	0.0
117	33.2	22.3	21.0	16.0	10.2		3.4	0.1	0.0
118	31.9	25.8	19.2	5.5	2.3		1.2	0.1	0.0
119	34.3	22.3	17.2	7.9	2.3		1.1	0.1	0.0
120	31.7	21.5	14.3	8.1	5.4		2.2		0.0
125	33.3	24.2	20.1	17.6	12.0		5.6	0.4	0.0
Avg.	32.5	23.7	18.4	11.5	6.9		2.9	0.1	0.0

* The value for each animal represents the percentage of initial radioactivity.

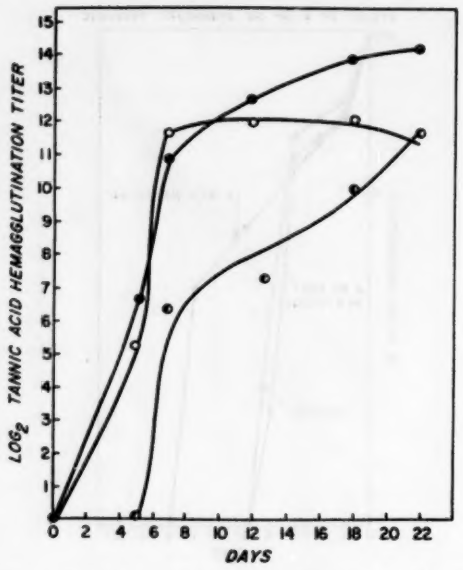

FIG. 6. PRODUCTION OF ANTIBODY DURING THE SECONDARY RESPONSE

○—○—○, control group; ●—●—●, 6-MP, 3 mg. per Kg. per day; ◑—◑—◑, 6-MP, 6 mg. per Kg. per day.

TABLE V

Percentage of globulin-bound antigen appearing during the secondary response

No.	Day after antigen injection					
	1	3	4	7	8	9
Control						
1	1.2	2.1	88.2	0		
2	1.6	1.9	93.1	0		
3	3.2	2.3	87.2	0		
5	4.8	3.7	67.8	0		
55	1.7	2.3	93.0	0		
57	6.7	4.1	0	0		
58	2.3	2.6	84.1	0		
59	1.6	3.8	0	0		
Avg.	2.1	2.9	64.0	0		
6-MP, 3 mg./Kg./day						
29	3.1	1.4	92.1	0		
30	1.6	2.8	91.7	0		
31	0.7	3.1	87.3	0		
32	1.4	0.5	94.6	0		
Avg.	1.7	1.9	91.4	0		
6 mg./Kg./day						
116	1.2	1.9	2.1	22.1	90.1	0
117	2.1	3.3	4.3	40.3	95.7	0
118	4.1	1.6	0.9	56.2	88.3	0
119	3.6	1.7	1.6	39.7	67.8	0
120	1.7		2.8	44.3	92.1	0
125	0.9	1.9	1.1	63.1	91.1	0
Avg.	2.1	1.9	2.2	45.9	87.3	0

TABLE VI

*Production of humoral antibody during the secondary immune response**

No.	Day after antigen injection					
	1	5	7	12	18	22
1	0	4.3	9.3	7.3	6.3	7.3
2	0	5.3	10.4	14.4	14.4	14.4
3	0	4.3	10.4	13.4	12.4	13.4
5	0	3.3	14.4	14.4	10.4	9.3
35	0	7.3	14.4	13.4	13.4	8.3
36	0	8.3	14.4	14.4	12.4	12.4
37	0	5.3	10.3	7.3	12.4	11.4
38	0	3.3	11.4	12.4	12.4	14.4
39	0	6.3	10.3	11.4	14.4	14.4
Avg.	0	5.3	11.7	12.0	12.1	11.7
Controls 3 mg./Kg./day						
29	0	4.3	8.3	14.4	14.4	13.4
30	0	6.3	9.3	13.4	12.4	14.4
31	0	7.3	11.4	10.4	14.4	14.4
32	0	8.3	14.4	12.4	14.4	14.4
Avg.	0	6.6	10.9	12.7	13.9	14.2
6-MP, 6 mg./Kg./day						
116	0	0	4.3	4.3	9.3	9.3
117	0	0	0	0	8.3	
118	0	0	14.4	14.4	14.4	14.4
119	0	0	12.4	14.4	14.4	14.4
120	0	0	7.3			
125	0	0	0	3.3	3.3	8.3
Avg.	0	0	6.4	7.3	10.0	11.6

* Titers for tannic acid hemagglutination tests; values are expressed as the $\log_2$ of the highest dilution giving a 1+pattern.

quickly rose and by the twelfth day they were at their peak.

6-MP, when given in a dose of 3 mg. per Kg. per day, had no effect on the secondary response. However, when twice that dose was given the onset of the immune disappearance phase occurred in the seventh day rather than on the third day. "Free" antibody appeared on the ninth day and the tannic acid hemagglutination titers rose thereafter to a peak on the twenty-second day after antigen injection. Although the animals of this group eventually attained antibody titers equal to those of the control group, the rate of increase was more gradual than in the untreated animals.

DISCUSSION

Antibody formation has been suppressed by a number of agents, including X-radiation (12), nitrogen mustard (13), thorium dioxide (14), cortisone (15), vitamin deficiencies (16), vitamin analogs (17), amino acid analogs (18), and purine antagonists (6, 19–21). Some of these,

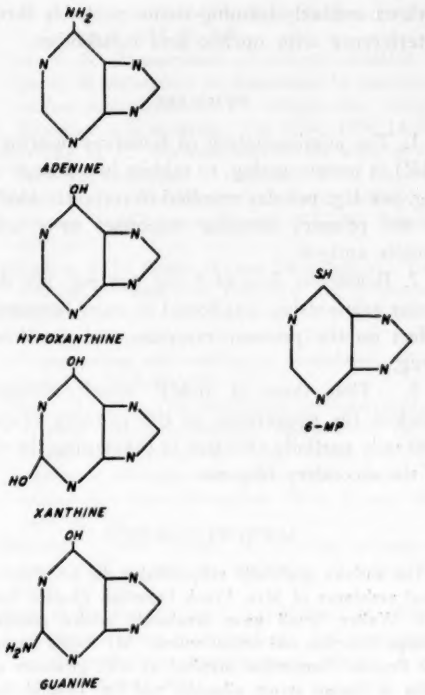

FIG. 7. STRUCTURAL FORMULAS OF 6-MP AND RELATED PURINES

such as X-radiation and nitrogen mustard, appear to exert their effects by direct destruction of antibody forming tissue; others, such as thorium dioxide, may act by inhibiting the function of the reticuloendothelial system. Metabolic antagonists of vitamins, amino acids and purines have striking effects on antibody production, but their mechanisms of action are poorly understood.

Although the effects of 6-MP (Figure 7) on immune responses are clearcut, its exact mode of action at the "biochemical level" is not well understood. There is much evidence to support the view that 6-MP acts as a nucleic acid antagonist, but the precise details of its action are ill defined. Its role in purine metabolism has been extensively investigated and it has been shown that 6-MP inhibits the incorporation of P^{32} into the nucleic acids of mouse tumors (22); not only is the *de novo* synthesis of nucleic acids by Sarcoma 180 cells suppressed by this agent (23), but radiolabeled 6-MP is probably synthesized into the nucleic acid molecules of certain cells (24). Other studies have demonstrated that the incorporation of hypoxanthine into nucleic acids of viruses is blocked by this agent (25). 6-MP probably acts as a nonspecific purine antagonist, since its inhibitory action on bacterial growth can be reversed by any of the natural purines (26). Work with 6-MP resistant mutants of *L. casei* suggested that 6-MP interferes with the conversion of adenine to guanine via hypoxanthine (27); this conclusion was later supported by experiments done with C^{14} labeled adenine which demonstrated that the 6-MP resistant mutant lacks a pathway for converting hypoxanthine to nucleic acid purines (28). Skipper came to similar conclusions concerning this action of 6-MP from his studies of the intestinal tissues of mice treated with this compound (23). 6-MP may also act on nucleic acid metabolism by interfering with normal purine interconversion mechanisms (29) or by competing with enzymes responsible for the conjugation of purine bases with ribose sugars, thereby forming ribosides and ribotides (30).

The widespread occurrence of purine moieties in nature has led to the investigation of the effect of 6-MP on systems other than nucleic acid metabolism. Thus, it has been found that the *in vitro* antimitotic activity of 6-MP can be overcome by coenzyme A, an adenine containing derivative of pantothenic acid essential in lipid metabolism (31); the increase in tissue diphosphopyridine nucleotide (DPN) that usually appears after the injection of nicotinamide into mice is prevented by treatment of these animals with 6-MP (32); amino acid (glutamate) incorporation into pea-seedling protein has been blocked with 6-MP (33); diminished amounts of adenosine triphosphate (ATP) have been found in tumor-bearing mice treated with this compound (34); and interference with hexokinase by 6-MP has been shown both *in vivo* and *in vitro* (35, 36). Whether the demonstrated effects of 6-MP on nucleic acid metabolism are the primary actions of this drug, or whether it acts through interference with other systems, such as ATP or hexokinase, and only secondarily induces biochemical lesions in nucleic acids, are speculative points. In any event, it is probable that the principle *observable* effect of 6-MP is on nucleic acid metabolism.

Certain conclusions can be drawn concerning the effect of 6-MP at the "immunological level."

It is possible to effect a complete block of the primary response to a soluble protein antigen with this agent. The linear relationship between the dose of 6-MP and its effect on antibody production suggests that the action of the drug in this regard might be that of a first-order chemical reaction, rather than that of a direct cytotoxic effect, as is seen with X-radiation or nitrogen mustard. If one assumes that the drug diffuses freely into all cells, and reaches approximately the same concentration in each antibody forming cell, then its action on antibody formation is probably not due to immediate death of antibody producing cells, since the dose-effect relationship is linear, rather than sigmoidal. This latter type of curve has been found by Makinodan and Gengozian (37) to express the effect of progressively increasing doses of X-radiation on immune responses in mice. The amount of 6-MP which effectively prevents antibody production during the primary response is only partially effective in preventing the appearance of a secondary response. The rapid onset and more intense cellular activity of antibody forming tissues which characterize the anamnestic response may also be accompanied by different metabolic patterns, either quantitative or qualitative, with different susceptibilities to 6-MP. An alternate explanation may be that these differences are a reflection of a smaller amount of drug available per cell during this phase of the immune response.

That 6-MP acts on antibody formation by nonspecific inhibition of protein production seems unlikely in view of the observation that the serum protein patterns of hyperimmunized animals treated with this drug are no different than those of control hyperimmunized rabbits (38). Furthermore, it has been shown that 6-MP does not affect non-neoplastic anabolism in man; the materials excreted during tumor shrinkage caused by the drug are derived solely from tumor destruction (39). Other experiments (40) have demonstrated that 6-MP does not interfere with the *in vivo* uptake of cellular antigens by the reticuloendothelial system, and it can be concluded that the drug does not suppress ingress of antigen into antibody producing cells.

In summary, then, the effects of 6-MP on both the primary and secondary inmmune responses may best be explained by a direct chemical attack on antibody forming tissue, probably through interference with nucleic acid metabolism.

SUMMARY

1. The administration of 6-mercaptopurine (6-MP), a purine analog, to rabbits in a dosage of 6-mg. per Kg. per day resulted in complete blocking of the primary immune responses to a soluble protein antigen.

2. Below the dose of 6 mg. per Kg. per day a linear relationship was found to exist between the effect on the primary response and the dose of drug.

3. That dose of 6-MP which completely blocked the appearance of the primary response was only partially effective in preventing the onset of the secondary response.

ACKNOWLEDGMENTS

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ION ASSOCIATION. III. THE EFFECT OF SULFATE INFUSION ON CALCIUM EXCRETION *†

By MACKENZIE WALSER and ANN A. BROWDER‡

(From The Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Md.)

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In the course of a study directed towards replacing extracellular chloride with sulfate in dogs, it was noted that sulfate infusion led to a considerable increase in the urinary excretion of calcium and signs of calcium deficiency (1, 2). The formation of a complex between calcium and sulfate was considered as a possible explanation of this observation, since it is well known that organic anions which complex calcium, including citrate (3) and ethylenediaminetetracetic acid (EDTA) (4), also augment calcium excretion. However, the nature of the association between calcium and sulfate in aqueous solution differs fundamentally from the covalent complexes formed with citrate and EDTA. It is believed that electrostatic forces alone are responsible for the apparently incomplete dissociation of calcium sulfate (5, 6). The physiological properties of this type of complex have not been previously studied.

This paper is one in a series directed towards this general problem. In the first paper (7) it was shown that the addition of multivalent anions (sulfate, phosphate or ferrocyanide) to serum led to an increased amount of complexed calcium in serum ultrafiltrate, determined by the difference between free calcium ion concentration (measured by the purpurate method) and total ultrafiltrable calcium. The results were consistent with the interpretation that calcium, when electrostatically paired with multivalent anions, does not combine with purpurate. In the second paper it was shown that calcium ions, when associated with sulfate, thiosulfate or dibasic phosphate, are not effective in sustaining cardiac contraction in the frog heart preparation (8).

In the present report it is demonstrated that urinary excretion of calcium is augmented by sulfate infusion and that this effect is far greater than during comparable diuresis induced by other agents. Wolf and Ball (9) observed increased excretion of calcium, magnesium and potassium during sulfate diuresis but found similar changes during infusion of 5 per cent glucose solution. The present data indicate that the action of sulfate in promoting calcium excretion is many times greater than can be attributed to urine flow alone. The interpretation that calcium is complexed by sulfate and thereby rendered less readily reabsorbed is shown to be consistent with the observations.

METHODS

Twenty-four experiments were performed on unanesthetized female dogs, connected to a constant infusion apparatus with a catheter in the jugular vein (10). This was inserted under local anesthesia with procaine hydrochloride. An indwelling catheter was inserted in the bladder and taped to the dog's back. The bladder was emptied every 15 to 40 minutes depending upon urine flow, but was not washed out. Venous blood samples were obtained in syringes containing heparin at approximately the mid-point of each period and were placed under oil. Plasma and diluted urine samples were stored in the frozen state until analyzed. Infusions were prepared from reagent grade chemicals dissolved in redistilled water and were warmed to prevent excessive cooling of the animal. The rate of infusion was controlled by a pump. Creatinine was administered in a priming dose followed by a sustaining infusion in all of the experiments. Fifteen experiments were performed in which sulfate was infused; in six, chloride was removed during the course of the experiment in order to achieve higher concentrations of sulfate in the plasma. This was accomplished by injecting intraperitoneally a volume of warmed fluid equivalent to 8 to 12 per cent of the animal's weight. This fluid was isosmotic and contained sodium sulfate as the major solute. One and one-half to two hours later, the peritoneal cavity was drained through a size 15

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‡ Work performed during tenure of a National Science Foundation student fellowship. Present address: Cornell Medical Division, Bellevue Hospital, New York, N. Y.

needle. The procedure was repeated as necessary to reduce the plasma chloride.

Infusion composition. The composition of the fluid employed for intravenous infusion was varied as follows: Even when relatively small amounts of sulfate were infused, the ensuing loss of calcium in the urine led to the development of hypocalcemia. Therefore, in most experiments calcium was added to the infusions either as calcium acetate or chloride. Occasionally this resulted in hypercalcemia, but these data have been omitted from the present paper. The remaining observations have been divided into two categories, those periods in which total plasma calcium was normal (2.25 to 2.75 mMoles per L., *i.e.*, 9 to 11 mg. per cent) and those with hypocalcemia (less than 2.25 mMoles per L.)

It was observed that sulfate infusion increased the urinary excretion of magnesium. Therefore experiments were performed both with and without the addition of $MgSO_4$ to the infusion. Hypermagnesemia was intentionally induced in some experiments, in order to counteract the symptoms of hypocalcemia. The results have been separated into periods with normal or reduced plasma magnesium (< 1.5 mMoles per L.) and those with hypermagnesemia (> 1.5 mMoles per L.), since magnesium is known to affect calcium excretion under certain conditions (11).

Potassium depletion also develops during infusion of large amounts of sodium sulfate (1). Therefore, K_2SO_4 was added to the infusions in amounts sufficient to offset urinary losses and to maintain plasma potassium concentrations within the normal range.

At moderate rates of infusion (< 10 ml. per minute) plasma bicarbonate and pH were not appreciably altered by infusions of mixtures of Na_2SO_4, $CaCl_2$, K_2SO_4 and $MgSO_4$. In the six experiments in which high plasma sulfate concentrations were produced by peritoneal dialysis, the development of moderate acidosis necessitated the administration of extra alkali, given as sodium acetate in the infusion.

The sodium concentration of isosmotic sodium sulfate solution is approximately 240 mEq. per L.; a sodium sulfate solution containing 140 mEq. of sodium per L. is markedly hypotonic (*ca.* 192 mOsm. per L.). Therefore, it is not possible to achieve a high plasma concentration of sulfate without inducing either hypernatremia or hypotonicity, unless a nonelectrolyte is also given. Rather than introduce the added complication of a nonelectrolyte such as mannitol, which would also act as an osmotic diuretic, we chose to compromise between hypernatremia and hypotonicity, permitting some degree of both. The infusions therefore contained sodium at concentrations of 160 to 200 mEq. per L., but were slightly hypotonic. The range of infusion concentrations studied for each of the other ions was as follows: potassium, 18 to 20 mEq. per L.; magnesium, 0 to 16 mMoles per L.; calcium, 0 to 5 mMoles per L.; sulfate, 95 to 120 mMoles per L.; acetate, 0 to 7 mMoles per L.; chloride, 0 to 10 mEq. per L. The effect of these infusions upon acid-base balance is of course unrelated to the pH of the solution infused but depends instead upon the buffer content, and upon secondary adjustments within the animal. The only buffer ion infused was acetate, which was included in four experiments at high plasma sulfate levels. When infused at less than 10 ml. per minute, the following solution was found to induce the least changes in the bicarbonate, potassium, calcium and magnesium concentrations of plasma: Na_2SO_4, 0.09 M; K_2SO_4, 0.008 M; $MgSO_4$, 0.003 M; $CaCl_2$, 0.003 M. It follows that this solution corresponds closely to the composition of the urine during prolonged sulfate diuresis.

In nine control experiments, either 0.16 M NaCl, 0.16 M $NaHCO_3$, 0.3 M sucrose or mixtures of these solutions were infused at rates varying from 5 to 30 ml. per minute, and the clearances of calcium and creatinine were determined. Since these infusions do not cause rapid depletion of calcium, magnesium or potassium, these cations were not added to the infusions.

The analytical methods employed were as follows: potassium, internal standard flame photometry; calcium, oxalate precipitation and permanganate titration (12, 13); magnesium, titan yellow (14, 15); chloride, amperometric titration (16); sulfate, benzidine precipitation and either titration of the precipitate (17) or colorimetric analysis of benzidine (18, 19); creatinine, alkaline picrate method (20); and protein, biuret method (21). With the calcium, magnesium and sulfate methods, the recovery of these substances added to plasma or urine was determined in each run. The mean recovery and probable errors of each method were as follows: calcium, 103 ± 3 per cent; magnesium, 104 ± 5 per cent; sulfate, 95 ± 6 per cent. Sulfate did not interfere with the determination of calcium or magnesium.

Calcium clearance was calculated without correction for protein binding.

RESULTS

The effect of rate of urine flow upon calcium excretion

In the lower portion of Figure 1 are shown the changes observed in calcium excretion during diuresis induced by infusions of NaCl, $NaHCO_3$ or sucrose. In the normal dog, calcium clearance is approximately 0.4 per cent of the glomerular filtration rate (22, 23). During administration of NaCl, $NaHCO_3$ or sucrose, this fraction increased to a maximum of 10 per cent or approximately twentyfold. This effect, although recognized qualitatively (9, 22), has not been previously documented in a quantitative manner. The interpretation of the effect of any agent upon the renal excretion of calcium without taking this phenomenon into consideration is hazardous.

The effect of sulfate infusion on calcium excretion

Two representative experiments are presented in Table I. In the first experiment (Dog F.) a

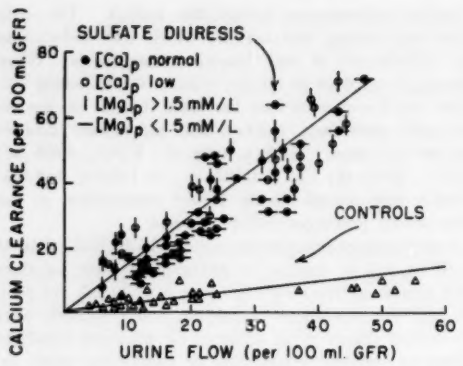

FIG. 1. CALCIUM CLEARANCE AS A FUNCTION OF URINE FLOW DURING INFUSION OF SULFATE

Control observations were obtained during infusion of NaCl, $NaHCO_3$ or sucrose.

sulfate solution containing calcium was infused for 74 minutes, at 10 ml. per minute. This resulted in a plasma sulfate concentration of 12.6 mMoles per L., and calcium clearances of 13 and 16 per cent of filtration rate. When calcium was omitted from the infusion, plasma calcium fell to 1.8 mMoles per L. (7.2 mg. per cent). Nevertheless, the calcium clearance ratio increased, concomitant with a rise in plasma sulfate to 19 mMoles per L. The concurrence of marked hypercalcuria and hypocalcemia suggests that the latter is due to the former, and indicates that the hypercalcuric effect of sulfate is pronounced.

In the experiment in Dog L., a considerably greater plasma sulfate level was achieved by employing repeated peritoneal dialysis. The dialysis fluid contained calcium, but the intravenous infusion fluid did not. The rate of infusion was adjusted to approximate the rate of urine flow, so that extracellular fluid volume was not greatly altered (except for the fluid in the peritoneal cavity). Plasma magnesium was progressively elevated to 3.8 mMoles per L. in order to counteract the symptoms of hypocalcemia. Plasma calcium fell to 1.3 mMoles per L. (5.2 mg. per cent) as plasma sulfate increased to greater than 44 mMoles per L. The calcium clearance ratio increased to 0.79, a value far in excess of any previously reported. With the exception of EDTA (22), no other agent has been reported to increase the calcium clearance ratio above 0.2. In the last period, the glomerular filtration rate fell to subnormal levels and the experiment was discontinued.

The results of all of the experiments are presented graphically in Figure 1. Calcium clearance is plotted as a function of urine flow, both parameters having been divided by filtration rate in order to minimize variation due to the size of individual dogs. Data obtained at subnormal filtration rates have been excluded (see below). Observations obtained during diuresis with NaCl, $NaHCO_3$ or sucrose are shown in the lower portion of the figure, and those with sulfate in the upper portion. Most of the values of urine flow per 100 ml. GFR greater than 25 were obtained from dogs subjected to peritoneal dialysis.

At comparable urine flows, calcium clearance is several times greater during sulfate diuresis than during diuresis induced by NaCl, $NaHCO_3$ or sucrose. The concentration of calcium in the urine remained relatively constant despite the great augmentation of urine flow, and as a result the relationship between calcium clearance and urine flow is approximately linear. The slope of the line indicates that the concentration of calcium in the urine is, on the average, one and one-fourth times the concentration of calcium in plasma. In the control studies with NaCl, $NaHCO_3$ and sucrose the ratio of urine calcium to plasma calcium concentrations averaged 0.23.

The data presented in Figure 1 also indicate that the effect of sulfate on calcium clearance is independent of the plasma concentration of calcium. The failure of hypocalcemia to affect this relationship emphasizes the obligatory nature of this action of sulfate. Furthermore, since hypercalcuria persists despite hypocalcemia, the effects cannot be attributed to the calcium which was infused in most of the experiments. The range of plasma calcium concentrations in the hypocalcemic animals was 1.2 to 2.25 mMoles per L., averaging 1.9 mMoles per L.

Magnesium was given in large amounts in some experiments to counteract the symptoms of hypocalcemia. It was important, therefore, to establish that the changes in calcium excretion were not the result of hypermagnesemia (range, 1.5 to 3.8 mMoles per L.). As indicated in Figure 1, the results were not significantly different in the presence of increased plasma magnesium, whether plasma calcium was normal or reduced.

TABLE I

The development of calcium deficiency during sulfate infusion

Time	Plasma Ca	Mg	Cl	SO_4	Urine Flow	GFR	C_{Ca}/GFR
min.	*mMoles/L.*				*ml./min.*		*ratio*
Dog F., 11.3 Kg.							
0	2.3	0.8			6.6	50	0.13
0	Start Infusion I, 7 ml./min.						
25–56	2.3	0.8			6.6	50	0.13
59–71	2.4	0.8		12.6	6.0	46	0.16
74	Start Infusion II, 10 ml./min.						
217–232	1.9	0.9		19.2	6.4	36	0.21
232–264	1.8	0.8			8.7	45	0.22
264–277	1.8	0.7		19.0	6.8	45	0.17
Infusion I: Na_2SO_4, 0.09 M; K_2SO_4, 0.008 M; $MgSO_4$, 0.002 M; $CaCl_2$, 0.002 M							
Infusion II: Same without $CaCl_2$							
	Ca	Mg	Cl	SO_4	Flow	GFR	
Dog L., 12.7 Kg.							
0	2.7	1.4	104				
0	Start Infusion I, 13 ml./min.						
30	Inject 1,750 ml. Solution B, intraperitoneally						
42–72	2.6		80		12.8	54	0.35
72–125	2.3		80		15.7		
120	Paracentesis, 900 ml. Inject. 1,000 ml. Solution B, intraperitoneally						
	Infusion I sped to 17 ml./min. Additional Infusion II, 0.5 ml./min.						
125–165	2.1		70	28	18.0	57	0.41
165–210	2.0		66		18.6	52	0.48
185	Infusion I sped to 20 ml./min.						
210–260	1.9	3.8	62		19.6	51	0.63
250	Paracentesis, 900 ml. Inject 1,300 ml. Solution B, intraperitoneally						
260–301	1.8		54	36	23.8	69	0.47
301–346	1.6		52	44	18.2	47	0.65
358	Paracentesis, 1,400 ml. Inject 1,600 ml. Solution B, intraperitoneally						
346–391	1.3		48		19.8	45	0.58
391–447	1.3	3.3	39		18.3	43	0.71
452	Paracentesis, 1,400 ml. Inject 1,400 ml. Solution B, intraperitoneally						
447–515	1.3		32		6.5	15	0.79
Infusion I: Na_2SO_4, 0.1 M; K_2SO_4, 0.004 M; NaAcetate, 0.0037 M							
Infusion II: $MgSO_4$, 0.16 M							
Solution B: Na_2SO_4, 0.1 M; CaAcetate, 0.005 M							

The lowest rates of sulfate infusion studied were equivalent to approximately 5 per cent of glomerular filtration rate. These infusions induced an increase in plasma sulfate from a normal value of 1 mMole per L. (in the dog) to approximately 6 mMoles per L. As Figure 1 shows, even these observations made during mild sulfate diuresis differ considerably from those obtained with NaCl, $NaHCO_3$ or sucrose.

Table II, discussed below, also shows the effect of a low rate of sulfate infusion on calcium excretion. The substitution of sulfate solution for saline at 5 ml. per minute led to a fivefold increase in calcium excretion.

The effect of hyperkalemia on the calciuretic action of sulfate

In most of the experiments potassium was administered in order to maintain a normal potas-

TABLE II

The effect of hyperkalemia on the calciuretic action of sulfate

Time	Plasma			Urine		C_{Ca}/GFR
	K	Ca	SO_4	Flow	GFR	
min.	*mMoles/L.*			*ml./min.*		*ratio*
Dog I, 8.9 Kg.						
0	Start Infusion I, 5 ml./min.					
49–81	4.7	2.4		3.5	38	0.03
92	Change to Infusion II, 5 ml./min.					
213–247	5.1	2.6	12.0	4.6	38	0.15
247–273	5.1	2.7	12.6	5.3	40	0.16
273	Additional Infusion III, 5 ml./min.					
297–309	7.6	2.4	12.3	7.8	45	0.19
309–330						
309–330				7.2		
330–352	8.3	2.5	12.4	6.9	44	0.17
352–372		2.4	11.8	7.0	46	0.16
372–399	9.5	2.3	11.8	6.0	43	0.17

Infusion I: NaCl, 0.16 M

Infusion II: Na_2SO_4, 0.09 M; K_2SO_4, 0.008 M; $MgSO_4$, 0.002 M; $CaCl_2$, 0.002 M.

Infusion III: KCl, 0.1 M

sium balance. In some instances this resulted in hyperkalemia. Three experiments were therefore conducted to determine whether excess potassium modifies the relationship depicted in Figure 1. The results in one experiment are presented in Table II. The infusion of KCl in amounts sufficient to increase plasma potassium concentration to 9 mEq. per L. did not alter calcium excretion appreciably. The results in the other two experiments were similar.

The role of filtration rate

The administration of NaCl or $NaHCO_3$ usually led to a rise in filtration rate. In the sulfate experiments, glomerular filtration rate usually remained within normal limits. In the six experiments in which peritoneal dialysis was performed to achieve higher plasma sulfate concentrations, filtration rate eventually fell to subnormal levels, but these data have not been included. The mean normal filtration rate in dogs is reported to be 4.3 ml. per minute per Kg. (24). The range of filtration rates observed in the sulfate experiments shown in Figure 1 was 3 to 7 ml. per minute per Kg. In this range, the calcium clearance ratio was not appreciably affected by variations in filtration rate.

The relationship between calcium excretion and sulfate excretion

In approximately half of the collection periods depicted in Figure 1, plasma and urine were analyzed for sulfate in order to define the relationship between calcium and sulfate excretion. There are a number of ways of expressing the results, *e.g.*, calcium excretion (or clearance) as a function of the sulfate level of plasma or the excretion of sulfate. Each of these yields a more or less

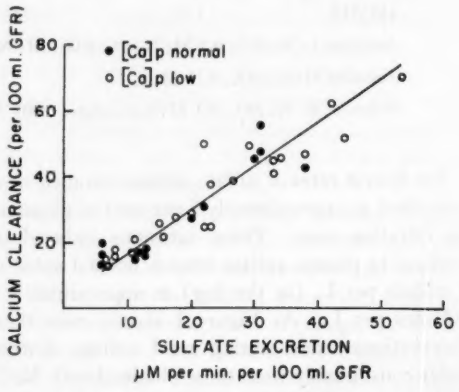

FIG. 2. CALCIUM CLEARANCE AS A FUNCTION OF SULFATE EXCRETION DURING SULFATE DIURESIS

linear relationship. The most useful appears to be that depicted in Figure 2, *i.e.*, calcium clearance as a function of sulfate excretion, both parameters having been corrected to 100 ml. of glomerular filtration rate. The straight line indicates that the ratio of calcium concentration in urine to that in plasma is proportional to the urine sulfate concentration. During sulfate diuresis, the urinary concentration of sulfate varied from 60 to 140 mMoles per L. but was unrelated to the rate of sulfate administration. The data indicate that each millimole of sulfate excreted is accompanied by 0.03 mMole of calcium, provided plasma calcium is normal, and correspondingly less during hypocalcemia.

Other effects of sulfate infusion

Sulfate appeared to be completely nontoxic to animals in whom deficiencies of calcium, magnesium and potassium were prevented by the addition of these cations to the infusions. In one animal weighing 17.9 Kg., a continuous intravenous infusion was maintained for four days during which 34 L. of distilled water was given, containing 435 Gm. Na_2SO_4, 44 Gm. K_2SO_4, 10 Gm. $CaCl_2$ and 16 Gm. $MgSO_4$. No significant change occurred in plasma calcium, magnesium or potassium concentrations or in the glomerular filtration rate; the dog's appetite and disposition were unaffected. The toxicity of sulfate described earlier by us (1) apparently represented magnesium deficiency, since it can be prevented by adding $MgSO_4$ to the infusions.

Measurements of the plasma sodium, potassium and chloride concentrations, osmolality, total CO_2 and pH were also made in most experiments. Usually, plasma sodium rose, chloride and osmolality fell, and potassium, CO_2 and pH remained normal. These data do not appear to have any direct bearing on the relationship under study here, and have therefore been omitted. Urine pH varied widely, but had no apparent effect on the calcium clearance ratios.

DISCUSSION

These observations indicate that the infusion of large amounts of sulfate in dogs leads to a marked increase in calcium excretion by the kidney. The urinary concentration of calcium remains higher than that of plasma despite profound diuresis. As a result, sulfate infusion leads to a negative calcium balance and, if given rapidly, to hypocalcemia. On the other hand, the infusion of saline or other osmotic diuretics leads to a fall in urinary calcium concentration and an increment in calcium excretion which is only one-fifth as great.

Since calcium clearance in these experiments was determined without correction for protein binding, it is conceivable that part of the increase in calcium excretion could be attributed to a reduction in the binding of calcium to plasma protein and a consequent increase in the calcium concentration in the glomerular filtrate. Ultrafiltrates were prepared from 32 of these plasma samples by the method of Toribara, Terepka and Dewey (25) and analyzed for calcium. The means (and standard errors) for the diffusible fractions of calcium in normal and sulfate-loaded dogs were 58 ± 6 and 75 ± 2 per cent, respectively. The average concentration of calcium in glomerular filtrate, relative to that in plasma, is therefore increased by (75–58)/58, or 29 per cent, during sulfate diuresis. However, calcium excretion was increased approximately fivefold during sulfate diuresis, as compared to saline diuresis, and this effect persisted despite marked hypocalcemia. Therefore the changes in calcium excretion cannot be attributed to altered binding of calcium to plasma protein. The significance of this change in protein binding is under study. Plasma protein concentration, determined in four experiments, either remained constant or decreased 10 to 20 per cent, a change far too small to account for the observed increase in the diffusible fraction of calcium.

The possibility that these effects of sulfate may be due to complex formation between calcium and sulfate may now be considered. The association between calcium and sulfate is known to be weak compared to that between calcium and citrate, for example. Dissociation constants at ionic strength 0.16 M are 0.06 M for calcium sulfate and 0.0006 M for the calcium citrate complex [(26), Vol. I, page 39, Vol. II, page 80]. Therefore the question arises whether the electrostatic complex between calcium and sulfate can be present in amounts sufficient to account for the present findings. This question is difficult to answer, since the concentrations of calcium and sulfate,

as well as the ionic strength of the tubular fluid, change progressively from glomerulus to the final urine. However, it is possible to estimate the fraction of urinary calcium electrostatically paired with sulfate. The concentration of sulfate in the urine is approximately 0.10 M during sulfate diuresis even at infusion rates of less than 5 ml. per minute. Since no other anion is present in significant concentrations during prolonged infusion of sulfate and since the predominant cations are monovalent, the ionic strength of the urine is approximately 0.3 M. Using a standard activity coefficient equation such as that of Davies [(6), Equation 9.13, page 230] the dissociation constant of calcium sulfate at this ionic strength becomes approximately 0.08 M. The ratio of free calcium ions to calcium complexed by sulfate can therefore be calculated as 0.08/0.10 or 0.8.

Thus approximately half of the calcium in the urine is electrostatically associated with sulfate.

There are no comparable observations to indicate what fraction of the urinary calcium is complexed by citrate or EDTA during infusion of these agents. It would be of interest to determine how the clearance of free calcium ions varies during administration of these substances as compared to sulfate. At present it can only be stated that the association of sulfate with calcium is the most likely explanation of its effect on calcium excretion, just as the complexing of calcium by citrate and EDTA is their probable mechanism of action. Each agent is effective at a concentration which reflects its affinity for calcium: EDTA in minute amounts, citrate in intermediate quantities, and sulfate in relatively large quantities. Sulfate is somewhat easier to employ for this purpose because the risk of tetany is less than with EDTA, and unlike citrate, it is not metabolized (*27*).

Apart from these considerations, the principal significance of this study lies in the demonstration that electrostatic complexes may affect renal excretion of ions. It is customary to consider the renal transport of individual ions as separate processes, in which the requirement of electroneutrality is fulfilled either by exchange with particles of like charge or by secondary movements of oppositely charged ions. The present studies indicate that, in addition, tubular transport may be modified by electrostatic interaction between ions of opposite charge. Thus metals cannot be considered as free, physiologically speaking, even though they are fully ionized. The calcium electrostatically paired with sulfate is believed to be ionized but yet undissociated. Its tubular reabsorption is apparently impaired. Other aspects of this phenomenon have been discussed elsewhere (*7*, 8).

SUMMARY

Intravenous infusion of sulfate in dogs leads to a marked increase in calcium excretion. Calcium clearance in relation to filtration rate was on the average five times greater than during comparable diuresis induced by NaCl, $NaHCO_3$ or sucrose. Rapid sulfate infusion led to hypocalcemia; nevertheless, hypercalcuria persisted. The data have been analyzed in terms of electrostatic association between calcium and sulfate ions in the renal tubular fluid. Half of the urinary calcium was estimated to be paired with sulfate ions. It is concluded that ion-pair formation may play a role in the renal transport of certain electrolytes.

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OBSERVATIONS ON DOUBLE ALBUMIN: A GENETICALLY TRANSMITTED SERUM PROTEIN ANOMALY * †

By DAVID P. EARLE, MARTIN P. HUTT,‡ KARL SCHMID AND DAVID GITLIN

(From the Department of Medicine, Northwestern University Medical School, Chicago, Ill., and the Departments of Medicine and Pediatrics, Harvard Medical School, Boston, Mass.)

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Knedel (3, 4) has published observations on "double albumin" noted on electrophoresis of the serum proteins of eight members of two families in Germany. The anomaly appeared in three generations of one of the families. Immunochemical studies demonstrated that both fractions in the albumin region, designated A_1 and A_2 by Knedel, were indeed albumin.

The present paper is a report of a similar anomaly in 25 of 43 members of a family of Norwegian descent living in Illinois. A number of immuno- and physicochemical observations on the anomalous protein are described.

In our preliminary report (1) we designated the two fractions A and B, being unaware at the time of Knedel's prior work and designations of fractions A_1 and A_2. Despite Knedel's priority, we have decided to retain our terminology of albumin A and albumin B. Albumin A corresponds to Knedel's fraction A_1 and probably is normal albumin, as will be shown. Albumin B corresponds to Knedel's fraction A_2, and is an anomalous albumin. No evidence exists that the anomalous albumin fractions in the several unrelated families are chemically identical. Paralbumin, therefore, might be a more acceptable general designation for the anomalous protein.

OBSERVATIONS

1. Discovery of anomaly, clinical and genetic data. A 22 year old white male complained of pain in the right costo-vertebral angle area. The urine contained 2 plus protein, rare red blood cells, 6 to 8 white blood cells per high power field and rare hyaline casts. The blood urea nitrogen was 42 mg. per 100 ml.; the urea clearance, 17 per cent of normal; and the phenolsulphonphthalein excretion, 5 per cent in 15 minutes. Total serum proteins were 7.2 Gm. per 100 ml.; the albumin, 5.2; and the globulin, 2.0 (by precipitation). The serum cholesterol was 222 mg. per 100 ml. The right kidney appeared to be normal on intravenous pyelograms but the left kidney and ureter were not visualized. On cystoscopy the right trigone was prominent but neither a left trigone nor a left ureteral orifice could be visualized.

We were very surprised to observe on the paper electrophoretic pattern of his serum proteins what appeared to be a double albumin (Figure 1). The persistence of this pattern has been demonstrated in four samples of serum obtained from this patient over a period of one year. A similar anomaly was demonstrated in his urinary proteins after concentration by dialysis (Figure 1).

On the thought that the protein anomaly and the absence of a kidney might be associated and transmitted genetically, we instituted a study of the patient's family. Sera from his mother and four of her siblings did not have the anomaly. However, study of the patient's father and his family was very rewarding, as shown by the

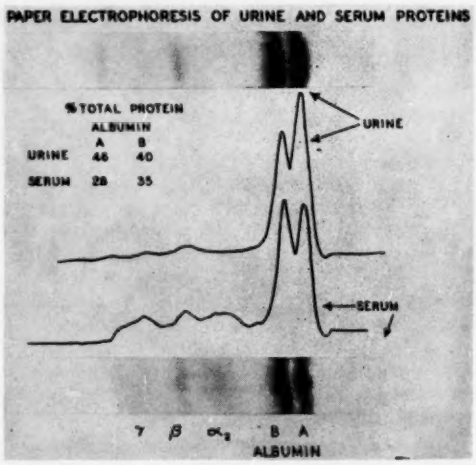

FIG. 1. PAPER ELECTROPHORETIC PATTERNS OF SERUM AND URINARY PROTEINS OF ORIGINAL SUBJECT WITH DOUBLE ALBUMIN

* Some of these data were presented at the annual meeting of the Association of American Physicians, May, 1958 (1), and at the International Biochemistry Congress of Vienna, September, 1958 (2).

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‡ Present address: Department of Medicine, University of Colorado Medical School, Denver, Colo.

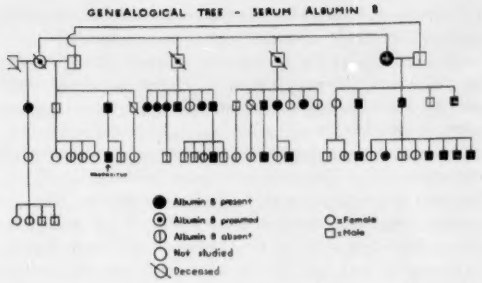

FIG. 2. GENEALOGICAL TREE OF FAMILY WITH DOUBLE SERUM ALBUMIN

genealogical tree (Figure 2). The anomaly, as indicated by solid dots (females) and squares (males), undoubtedly resides in his paternal grandmother's family. The deceased paternal grandmother had affected offspring by two different mates. Her sister, still alive, has the double peak, while her two deceased brothers presumably had the anomaly since it appeared among their descendents. Finally, neither the paternal grandfather nor his brother (brothers married sisters) have the double peak (Figure 2). The paternal grandmother and her sister were the children of immigrants to Illinois from Norway.

So far as can be determined, the anomalous albumin in this family is not associated with disease except incidentally. Intravenous pyelograms were obtained in six affected members of the family; the original subject is the only one with congenital absence of a kidney. Furthermore, no other affected member of the family has significant proteinuria. A fairly high incidence of arteriosclerotic heart disease was noted in the family, but individuals without the protein anomaly did not differ in this respect from members with the anomaly.

The fact that eight offspring of known negative parents are not affected supports the assumption that unaffected members of the family cannot pass the anomaly to their children. The latter, therefore, are excluded from the "genetic risk" group in Table I. On the other hand, three deceased members of the first generation whose offspring had the anomaly are assumed to have had the anomaly and, therefore, they and all their offspring are included in the "genetic risk" group. Two offspring of parents not studied and without known anomalous offspring, as well as 11 collateral relatives, are not affected. On the basis of these assumptions, 28 of 46 exposed to the genetic risk are affected (Table I).

TABLE I

Incidence of double albumin in family

	Total subjects	With double albumin
Exposed to genetic risk	46	28
Offspring of negative parents	8	0
Offspring of parents not studied and without other children	2	0
Collateral relatives	11	0

TABLE II

Serum proteins and cholesterol in family with double albumin

	28 Normal subjects	25 Affected subjects
$\frac{\text{Albumin B}}{\text{Total albumin}} \times 100$	0	57±4.4*
$\frac{\text{Total albumin}}{\text{Total protein}} \times 100$	49	50
Total protein (Gm./100 ml.)	7.5	7.6
Total serum cholesterol (mg./100 ml.)	259	268

* If alpha 1 globulin (which is obscured by albumin B) were deducted, this figure would approximate 50 per cent.

The relative amounts of albumins A and B are remarkably constant (Table II). Albumin B makes up on the average 57 per cent of the total serum albumin, with a standard deviation of 4.4 per cent. If the usual normal value for alpha 1 globulin (which usually is obscured on paper electrophoresis by albumin B) were deducted, albumin B would account for approximately half the total serum albumin of the anomalous subjects. The sum of albumins A and B is equal to the normal serum albumin value, while the total serum protein value in the anomalous subjects likewise is normal. In anomalous subjects, therefore, the extra albumin fraction apparently replaces an equivalent amount of normal albumin.

Total serum cholesterol values are slightly greater in the anomalous subjects than in the normal members of the family, but the difference is not statistically significant.

2. Immunochemical observations. Materials and methods: Sera from five individuals with the anomalous serum protein, from two of their siblings without the anomaly, and from three normal persons unrelated to the pedigree were selected for immunochemical studies.[1] The proteins of the sera were first separated by zonal electrophoresis in both powdered starch (5) and agar gel (6) at pH 8.6 in diethylbarbiturate-citrate buffer having an ionic strength of 0.1. Electrophoresis was performed at 4° C. using 8 to 10 volts per cm. of length of starch or agar for 24 hours. In some instances, a crystal or two of bromphenol blue was dissolved in a serum before placing the serum in the starch or agar. Under these circumstances the separation of the albumins, identified by means of the bound blue dye, could be observed during electrophoresis (Figure 3). On other occasions, the localization of the proteins in the starch or agar was determined by

[1] We are greatly indebted to Dr. Dale Learned of Kankakee, Ill., for his generous assistance in obtaining large amounts of blood for these studies.

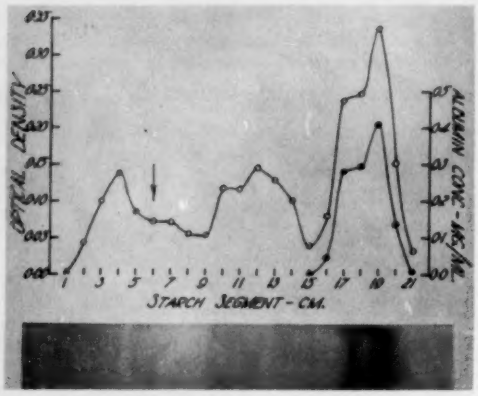

FIG. 3. STARCH ELECTROPHORESIS (○—○—○) OF SERUM FROM PROPOSITUS, AND ALBUMIN CONCENTRATIONS (●—●—●) ESTIMATED WITH RABBIT ANTI-ALBUMIN ANTISERUM

Arrow indicates origin. Optical density = O.D. $\frac{1\text{ cm.}}{280\ m\mu}$. Below graph is a photograph of the starch block showing binding of bromphenol blue by both albumins. Anode is toward right side of figure.

pressing a dry strip of Whatman No. 1 filter paper against the block. The wet paper strips were then stained in 1 per cent bromphenol blue (in 95 per cent ethanol saturated with mercuric chloride) and finally washed in tap water, the result being a "print" of the protein distribution in the block.

For electrophoresis, two different starch preparations were used from which little or no ultraviolet light absorbing material was released with pH 8.6 barbiturate-citrate buffer or with 0.15 M NaCl. One batch was prepared by repeated washing of potato starch (Fischer, iodometry grade) with distilled water followed by several washes each of pH 8.6 barbiturate-citrate buffer, 0.1 M pH 8.6 borate buffer, distilled water and finally acetone. The starch was dried in air at room temperature. The other starch preparation was obtained from Connaught Laboratories (Toronto) as a hydrolyzed derivative for use in forming starch gels (7). In this study, however, the starch was used only as a powder and proved admirably suited for this purpose. The starch blocks measured 4 by 30 cm., were covered with wax paper and supported on individual one-fourth inch thick plate glass strips. After electrophoresis, the starch blocks were cut into 0.5 or 1.0 cm. segments. The serum proteins were eluted with 0.15 M NaCl. The optical densities of the eluates were measured in a Beckman spectrophotometer at a wave length of 280 mμ. When bromphenol blue had been dissolved in the sera, the eluates were measured at 600 mμ as well. At the latter wave length, the blue color of the dye complex was the principal absorbant. Where applicable, as for the eluted albumins from sera without added dye, the extinction coefficient for albumin $E^{\frac{1\text{ per cent}}{1\text{ cm., }280\ m\mu}}$ was taken as 5.32 (8).

Aliquots of those eluates representing the central starch segment for each albumin peak were appropriately diluted with 0.15 M NaCl so that a series of concentrations were obtained for each albumin. Antigen-antibody reaction mixtures were prepared by adding 1.0 ml. of a given albumin dilution to 1.0 ml. aliquots of rabbit antiserum *vs.* normal human serum albumin prepared as described elsewhere (9). The mixtures were incubated at 37° C. for one hour and then at 4° C. for 20 hours. The resulting specific precipitates were collected by centrifugation at 3,000 rpm at 0° C. for 15 minutes and washed twice with cold 0.15 M NaCl. The amount of specific precipitate was measured by a spectrophotometric method (10). The quantitative precipitation curves obtained by plotting the amount of specific precipitate *vs.* the relative amount of albumin added to the antiserum were compared as described previously (9). The concentration of albumin in all the eluates was estimated by the same precipitation method, the rabbit antiserum being standardized with crystallized pooled human serum albumin, preparation Decanol 10 (8), the antigen against which the antiserum was made.

The immunochemical characteristics of the albumins separated by starch electrophoresis were compared with each other and also with pooled normal albumin by means of the two-dimensional agar diffusion method of Ouchterlony (11). For this purpose, 2 per cent agar in 0.1 M, pH 8.6 borate buffer was poured into Petri dishes to a depth of 4 to 5 mm. Seven holes, each with a diameter of approximately 7 mm., were cut through the agar with a cork borer. The bottoms of the holes were sealed with fresh agar. Six of the wells were situated in a circle approximately equidistant from each other and from the seventh central well (Figures 4 and 6). The central well was filled with rabbit or horse antiserum *vs.* normal human serum albumin. One of the peripheral wells was

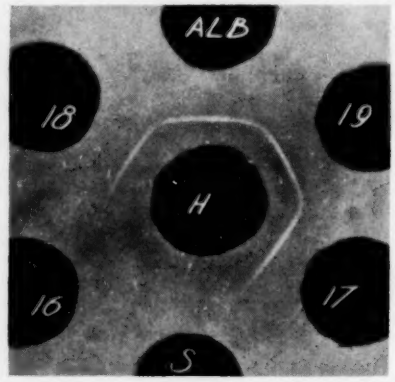

FIG. 4. AGAR GEL ANTIGEN-ANTIBODY DIFFUSION REACTIONS USING HORSE ANTI-ALBUMIN IN THE CENTRAL WELL (H) AND NORMAL HUMAN SERUM ALBUMIN (ALB) AND 0.15 M NaCl (S) AS CONTROLS

Numbers in wells refer to the number (cm.) of segment in Figure 3 from which eluates were obtained.

filled with 0.1 per cent crystallized pooled normal human serum albumin in 0.15 M NaCl and the opposite well with 0.15 M NaCl. Each of the remaining wells was then filled with an eluate from the starch segments. The horse antiserum used has been described elsewhere and is that preparation designated as Bleeding No. 3 (12). The Petri dishes were kept in moist chambers at room temperature and examined daily. The reader is reminded that in such agar diffusion systems, a band of antigen-antibody precipitate forms in the agar gel between the wells of antigen and antiserum. If a band of specific precipitate forms between the antiserum well and one antigen well and this crosses but does not merge with that formed between the same antiserum and a second antigen well, forming an X, then the reacting antigens in the two wells are immunochemically different. On the other hand, if the *end* of one precipitation band merges into the *side* of the other, forming a Y, then the two antigens are considered to have some haptenic groups in common. If one end of each precipitation band merges completely into the other to form a V, the two antigens under proper conditions are at least qualitatively identical as regards to their haptenic groups; the latter reaction is termed an "identity" reaction in this report.

The agar gels used for electrophoresis were prepared on $\frac{1}{4} \times 8 \times 10$ inch or $\frac{1}{8} \times 6 \times 8$ inch pieces of plate glass, the gel block being divided into four or five channels of equal width by longitudinal troughs or wells. The details of this preparation of agar plates have been presented elsewhere (13). After electrophoresis, horse antiserum *vs.* normal human serum albumin was placed in the wells and the plates kept in moist chambers at room temperature. In 24 to 48 hours, curvilinear bands of specific precipitate appeared where anti-albumin antibodies diffusing into the agar reacted with human serum albumin.

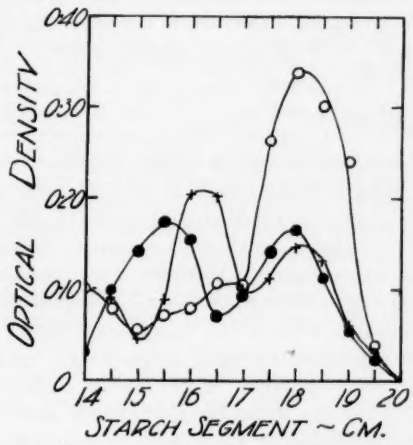

FIG. 5. STARCH ELECTROPHORESIS OF SERA FROM TWO PERSONS WITH DOUBLE ALBUMIN (F = ●—●—●, V = +—+—+) AND OF SERUM FROM A NORMAL RELATIVE (C = ○—○—○)

Only a part of each electrophoretic pattern is shown.

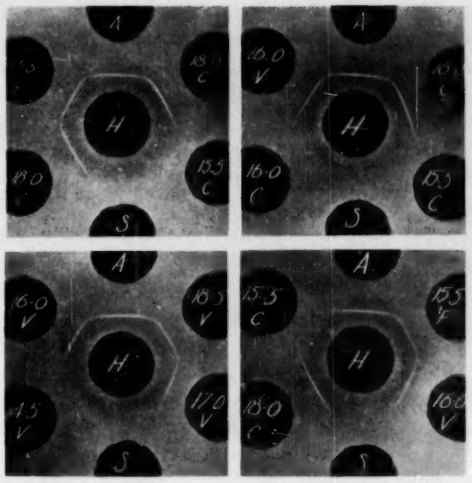

FIG. 6. AGER GEL ANTIGEN-ANTIBODY DIFFUSION REACTIONS USING HORSE ANTI-ALBUMIN (H), WITH NORMAL ALBUMIN (A) AND 0.15 M NaCl (S) AS CONTROLS

Numbers in wells refer to the number (cm.) of segment in Figure 5 from which eluates were obtained. The letters below the numbers refer to the subjects in Figure 5 (C = normal relative, V and F = individuals with double albumin).

RESULTS

The extent of separation of albumin A and B, obtained by starch electrophoresis, is shown in Figures 3 and 5. The precipitation curves obtained by reacting rabbit anti-albumin antiserum with various dilutions of these albumins in the zones of antibody excess and equivalence were indistinguishable from each other and from that obtained by reacting the antiserum with normal human serum albumin. The immunochemical estimations of albumin in the eluates from the starch segments paralleled the optical densities for the albumin peaks as shown in Figure 3. When bromphenol blue was omitted from the sera, the concentration of albumin in eluates from the central portion of the albumin peaks as estimated from optical density was within ±5 per cent of the concentration determined with rabbit antiserum.

The results obtained when eluted proteins from starch electrophoresis were reacted with horse anti-albumin antiserum in agar gels are indicated

in Figures 4 and 6. It may be noted that: *1*) No albumin was detected in segments from normal human sera analogous to those in which albumin B was found in anomalous sera (Figure 6, top left, Wells 15.5-C and 15.5-F; top right, Wells 16.0-C and 16.0-V). *2*) The precipitation bands of albumin A and B merged into each other without crossing, representing an "identity" reaction (Figure 4, Wells 17 and 19). *3*) Albumin B gave an "identity" reaction with serum albumin from a normal sibling (Figure 6, top left, 15.5-F and 18.0-C). *4*) Albumin B from two different individuals gave "identity" reactions (Figure 6, bottom right, Wells 15.5-F and 16.0-V). *5*) All albumins gave "identity" reactions with pooled normal human serum albumin. Similar results were obtained with rabbit anti-albumin antiserum.

Agar gel electrophoresis of normal human serum followed by diffusion of horse anti-albumin antiserum into the gel resulted in a curved precipitation band with a single vertex (Figure 7, A and D). Sera containing albumins A and B gave a single continuous curvilinear precipitation band with *two* vertices (Figure 7, B, C and E).

Free electrophoresis

Free electrophoresis in the Tiselius apparatus of serum and plasma from anomalous subjects (Figure 8) confirmed the paper electrophoretic data. The double peak in the albumin region is apparent at pH 8.6. Albumin A mobility is similar to that of normal human serum albumin.

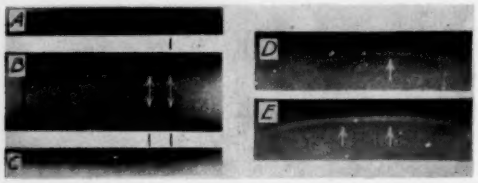

FIG. 7. AGAR GEL ELECTROPHORESIS OF SERUM FROM A NORMAL PERSON (SECTION A) AND OF SERA FROM TWO PERSONS WITH DOUBLE ALBUMINS (SECTIONS B AND C)

Only the lower half of agar Section A and the upper-half of agar Section C are shown. Horse antiserum had been placed in wells between Sections A and B, and B and C after electrophoresis. The precipitation band seen in C is enlarged in E and that from the upper half of A is enlarged in D. The lines between the agar sections and the white arrows indicate vertices of the precipitation bands. Anode is to the right of the figures.

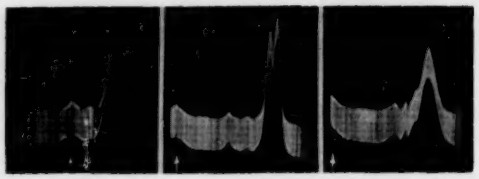

FIG. 8. MOVING BOUNDARY ELECTROPHORESIS AT VARIOUS PH'S OF FRACTION IV + V OBTAINED FROM SERUM OF THE PROPOSITUS

Left: pH 3.5, acetate buffer; middle: pH 8.6, diethylbarbiturate-citrate buffer; right: pH 12.2 glycine-NaOH buffer. Descending patterns; anode to left at pH 3.5 and to right at pH 8.6 and 12.3. Arrows indicate buffer anomaly.

Comparison of migration of I^{131} labeled human serum albumin with albumins A and B

I^{131} labeled human serum albumin mixed with sera of anomalous subjects migrated upon paper electrophoresis with fraction A rather than fraction B.

Ultracentrifugation

The two albumin fractions could not be separated by ultracentrifugation of plasmas from affected individuals. Likewise, Cohn Fraction IV + V from two abnormal sera did not reveal any variations from normal when studied by ultracentrifugation in a Spinco ultracentrifuge, Model E, at approximately 250,000 times gravity in 0.15 M saline. Albumin B, therefore, is not a polymer of albumin A nor does it represent a fraction of it.

Solubility

When an anomalous serum was fractionated according to Cohn's method 10 (14) three fractions resulted: Fractions I + II + III, IV + V and VI. Fraction IV + V contained the major part of the albumin. However, Fractions I + II + III and VI, respectively, contained small amounts of albumin too, which split into A and B in approximately equal amounts as judged by paper electrophoresis at pH 8.6 in barbiturate-citrate buffer. It can be inferred from these data that the solubilities of albumin A and B in this system must be almost identical. Thus, the substitution of amino acid residues containing carboxyl groups in albumin A by other residues to form albumin B (*vide infra*) does not affect solubility.

Starch column electrophoresis and subfractionation with cationic detergent [2]

Subfractionation by the cationic detergent method of Jacox (15) revealed no differences between albumins A and B separated by starch column electrophoresis.

3. Electrophoretic studies at several pH's. Methods: The relative electrophoretic mobilities of albumins A and B were studied at various pH's using paper and moving boundary electrophoresis. When paper electrophoresis was employed, I^{131} labeled normal human serum albumin, prepared as described elsewhere (16), was mixed with each serum before applying the serum to the paper. This facilitated the identification of albumin A since albumin A and radioiodinated normal serum albumin migrated together. The weight ratio of radioiodinated albumin to the total albumin of the serum was less than 1:100 and the amount of radioactivity in the volume of serum sample used for electrophoresis, 5 to 10 μl., was approximately 0.1 μc. After electrophoresis the paper strips were stained with bromphenol blue, dried and scanned in a photometer for color intensity. The strips were then cut into 2.5 or 5 mm. wide segments perpendicular to the axis of migration and the segments assayed for radioactivity in a well-type Na I-crystal scintillation counter which gave approximately 1×10^6 counts per μc. of I^{131}. Normal sera and crystallized human serum albumin also were electrophoresed under the same conditions as the controls.

For moving boundary electrophoresis, the Perkin-Elmer instrument, Model No. 38, was employed. The sera were first fractionated by the low temperature ethanol-water method of Lever and co-workers (14). Fraction IV + V, after lyophilization, was used. With this fractionation method, over 90 per cent of the total albumin of each serum, as determined electrophoretically and immunochemically, appeared in Fraction IV + V. In the case of anomalous sera, the ratio of the two albumins as determined electrophoretically was the same in Fraction IV + V as in the original whole serum. The total albumin in Fraction IV + V represented approximately 80 to 85 per cent of the proteins in the fraction.

Electrophoresis was carried out over a pH range from 3.0 to 12.3. All buffers were of ionic strength 0.1. The following were used: phosphate, pH 3.0; acetate, pH 3.5 to 5.8; cacodylate, pH 7.0; phosphate, pH 7.0 to 12.0; barbiturate-citrate, 8.6 to 10.1; and glycine-NaOH, pH 9.6 to 12.3.

RESULTS

Between pH 3.5 and 12.2, albumin A had the same electrophoretic mobility as normal serum albumin. Between pH 4.5 and 10.1 inclusive the net charge on albumin A was always negative relative to the net charge on albumin B. Within this pH range, when both albumins had net positive charges, albumin B migrated toward the cathode more rapidly than albumin A, and when both albumins had net negative charges, albumin A migrated toward the anode more rapidly than albumin B. Between pH 11.6 and 12.2 and at pH 3.5, albumins A and B had the same mobility as evidenced by a single albumin peak in the anomalous sera, or in Fraction IV + V obtained from the anomalous sera. In Figure 8 are shown representative descending patterns obtained with moving boundary electrophoresis of Fraction IV + V from a single anomalous serum. The small peak on the Σ-anomaly side of the albumin peak at pH 12.2 (Figure 8) was not a specific characteristic of albumins A or B alone, since crystallized normal serum albumin acquired an identical irregularity at this pH. The other small peaks in Figure 8, aside from the Σ-anomaly, were attributable to proteins other than albumin in Fraction IV + V (14).

The changes in the *relative* mobilities of albumins A and B observed were *not* attributable to the use of different buffers at different pH's. Thus, in phosphate or glycine buffer below pH 11, albumins A and B had different mobilities, but at pH 11.6 in the *same* buffer systems, both albumins migrated together. Similarly, in acetate buffer at pH 4.5 the two albumins had different mobilities, but at pH 3.5 in the same buffer the albumins had the same mobility.

After electrophoresis at those pH's where both albumins migrated together, it was possible when using the moving boundary method to recover the sample of Fraction IV + V from the electrophoresis cell, redialyze it and then re-electrophorese it at pH 8.6. This was done to demonstrate that any effect of the previous high or low pH on the mobilities of the albumins was reversible. In each case, the double albumin peaks reappeared.

DISCUSSION

Our observations on "double albumin" in 25 of 43 members of a family of Norwegian extraction confirm and extend those of Knedel (3, 4), who described this anomaly in two families, and Nennstiel and Becht (17), who described one family. Dr. Knedel (18) informs us that he has found

[2] We are greatly indebted to Dr. Ralph F. Jacox for performing these studies.

a similar serum protein anomaly in another family as have Bennhold, Ott and Scheurlen more recently (19). All the families reside in the same area in Germany but are not related so far as is known. Weiner (20) has discovered a similar double albumin in six members of a large family of Italian (Salerno) extraction living in New York City. In all, therefore, seven families with double albumin have been discovered to date.

The data from the family described in this report leave little doubt that the anomaly is transmitted genetically. Each anomalous individual is heterozygous for the characteristic, having received a normal gene from one parent and an anomalous gene from the other. All unaffected persons of the family probably have received a normal gene from each of their parents. Penetrance appears to be complete since double albumin has been found in individuals ranging from one and one-half to 68 years of age.

No known marriages between two persons having the anomaly have occurred, so there has not been an opportunity to observe the effect of the anomalous gene in the homozygous condition. However, our observations of heterozygous individuals suggest the use of the term "codominant" because the effect of each gene, the normal and the anomalous, is seen in the heterozygotes.[3] Further, in the anomalous subjects albumins A and B each make up approximately half the total serum albumin and their sum is equal to the normal serum albumin level. Knedel (18), however, reports in his families that ratios of A to B of 3 : 2, 2 : 3, 2 : 1, 1 : 2 and 1 : 1 have been found. Nennstiel and Becht (17) report more B and A. The differences of the relative proportions of albumins A and B in various families probably can be explained by the relative effectiveness of the mutant gene in different families. Various other anomalies are inherited in a similar manner, as has been demonstrated particularly well in the inheritance of abnormal hemoglobin types (21). The extensive observations in our family, and those of the other families with this anomaly, indicate that double serum albumin is not associated with disease.

In view of the immunochemical data, little doubt exists that the protein designated as B in this report is indeed an albumin. The reactivity of fraction B with rabbit and horse antisera was the same quantitatively and qualitatively as that of albumin A and of normal serum albumin. Consequently, both the nature and number of haptenic groups of these albumins appear to be identical. Knedel (3, 4) reports that his fraction A_2 (corresponding to albumin B) on immunochemical and immunoelectrophoretic study appears to be an albumin. Albumin B behaved exactly as did normal serum albumin electrophoretically, immunochemically and ultracentrifugally. While the evidence available to date is not conclusive, it strongly suggests that albumin A *is* normal serum albumin. In accord with this, preliminary studies indicate that albumin A and normal serum albumin are indistinguishable as regards chromatography on carboxymethyl cellulose columns and as regards the binding of certain dyes. Albumin B, however, is quite unlike normal serum albumin or albumin A under these conditions. From this evidence, albumin B appears to be an anomalous serum protein. Its detection in certain sera is *not* simply due to an increase in the concentration of a protein normally present in the serum in small amounts. By sensitive immunochemical methods, albumin could not be found in normal serum in the albumin B electrophoretic position.

The change in the electrophoretic mobility of a protein that occurs with a change in pH is due to an alteration in the net charge on the protein molecules and is the result of the titration of the various hydrogen-ion binding groups of the molecules of protein at different pH values (22). In the pH range from 10 to 11.3, where the net charge on albumin B becomes equal to that on A, the groups that are titrated, and hence change in charge, are (23) : *1*) the ε-amino groups of lysine residues, *2*) the free sulfhydryl groups, and *3*) the phenolic hydroxyl groups of tyrosine residues.[4] As titration proceeds towards increasingly alkaline pH, the lysine residues lose their positive charge and the sulfhydryl and tyrosine residues gain a negative charge. In the acid pH range the free carboxyl groups lose their negative charge but the ε-amino group of lysine remains positively charged. The

[3] We are greatly indebted to Dr. Herman M. Slatis of Argonne National Laboratory for his helpful advice on our genetic data.

[4] Although the guanidinium groups of arginine residues may begin to titrate at the alkaline end of this range, their titration continues well beyond this range.

TABLE III

*Charge contributed to serum albumin by certain titratable groups in various pH ranges**

Group	Charge at pH 3.0 to 3.5	Charge at pH 4.5 to 10	Charge at pH 11.3 to 12.3
εNH_2 (lysine)	+	+	0
Guanidinium (arginine)	+	+	+–0†
Imidazolium (histidine) and α-NH_2	+	+–0†	0
Phenolic hydroxyl (tryosine) Sulfhydryl (cysteine)	0	0	
Carboxyl (aspartic, glutamic acids and α)	0		

* Adapted from Reference 23.
† Charge changes within range given.

observed changes might suggest a substitution of tyrosine, cysteine or lysine residues in albumin B for an equal number of carboxyl residues in albumin A. Reference to Table III suggests that such substitutions would result in the same net charge on the two albumins above pH 11.3, where they would have the same electrophoretic mobility. Between pH 4.5 and 10, albumin A would be negatively charged relative to albumin B. These substitutions could explain the observed difference in electrophoretic mobility between albumins A and B. The question of whether or not additional structural differences exist between albumins A and B (such as additional substitution of uncharged amino acids in B for other uncharged amino acids in A) can only be answered by analysis of these fractions by methods other than those herein described. On the other hand, it can also be noted from Table III that simple substitution of free carboxyl residues in A by lysine residues in B would not give equal charges and mobilities at pH 3.5 and 11.6.

SUMMARY AND CONCLUSIONS

1. A serum protein anomaly, double albumin, has been found on electrophoresis of the serum proteins in 25 of 43 members of a family, and is presumed to have been present in three deceased members of the family.

2. The anomaly has been observed in the heterozygous state. It is transmitted as a codominant characteristic with complete penetrance.

3. Anomalous albumin B is assumed to be the result of a mutation of a gene responsible for the synthesis of normal serum albumin.

4. The anomaly is not associated with disease.

5. The anomalous fraction replaces one half of normal serum albumin, and by immunochemical evidence is found to be an albumin.

6. Data obtained by electrophoresis at several pH's suggest that anomalous albumin B contains tyrosine, cysteine or lysine residues substituted for an equal member of carboxyl residues in normal albumin A.

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HUMAN PANTOTHENIC ACID DEFICIENCY PRODUCED BY OMEGA-METHYL PANTOTHENIC ACID*

By ROBERT E. HODGES, WILLIAM B. BEAN, MARGARET A. OHLSON AND ROBERTA BLEILER

(From the Metabolic Section of the Department of Internal Medicine, University Hospitals, College of Medicine, State University of Iowa, Iowa City, Iowa)

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Since the discovery that pantothenic acid comprised a major portion of coenzyme A, this vitamin has been studied intensively by workers in biology and nutrition as well as in clinical medicine. It was found to be essential for the growth, health and reproduction of many species of fowls and mammals. Its importance in human nutrition remained long in doubt, however, because of its presence in nearly all foods eaten by man. In 1954 we reported that pantothenic acid was essential in human nutrition. This study and subsequent ones demonstrated that young men fed a purified, partly synthetic diet deficient in pantothenic acid, became ill after about 12 weeks (1, 2). By adding omega-methyl pantothenic acid to the deficient diet, we were able to induce illness in about four weeks (3–5). The details of this illness and of the unpredictable results stemming from unforeseen peculiarities of the test program have been described (4–6). After correction of defects in the experimental plan, we established the characteristics of pantothenic acid deficiency in man. The symptoms included fatigue, headache, weakness, emotional lability, impaired motor coordination, paresthesias, muscle cramps and gastrointestinal disturbance such as nausea, vomiting and abdominal cramps. Some subjects had tachycardia, orthostatic hypotension and fluctuations in arterial blood pressure. The subjects developed an increase in the erythrocyte sedimentation rate, eosinopenia and an inability to respond to adrenocorticotropic hormone with further eosinopenia. They became increasingly sensitive to the hypoglycemic effect of insulin. Interpretation of these results was modified with each successive study. In some respects the findings suggested the same adrenocortical insufficiency which occurs in animals deficient in pantothenic acid, yet many features were missing from the human counterpart. Whatever the mechanism, our subjects were ill as a result of pantothenic acid deficiency. Most recovered promptly when large doses of this vitamin were given.

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METHODS

Four healthy young men were selected from prison volunteers. They ranged in age from 22 to 29 years, had been healthy all their lives, and were normal by physical examination (Table I). Their blood, urine, X-rays of the chest and electrocardiograms were normal. All were fed a general diet containing 3,200 calories and 120 Gm. of protein throughout the study. Calculations of the amount of pantothenic acid in this diet averaged 8.484 mg. daily (Table II). The regimen for each man differed from that of his fellows only in the amount of the anti-vitamin which he received. One man received none, another got 0.5 Gm. daily, a third 1 Gm. daily, and the fourth 2 Gm. daily. At the beginning of the seventh week of the test, the dose of the antagonist was doubled so that Subject No. 2 received 1 Gm.; No. 3, 2 Gm.; and No. 4, 4 Gm. daily. This schedule was maintained into the twelfth week when it was discontinued. Thereupon 4 Gm. of pantothenic acid was given to each man every day.

The men were under constant supervision. They were interviewed daily by R.E.H. or W.B.B. We made routine observations of their general condition, blood pressure, pulse and respiratory rates. They were weighed daily. Complete collections were made of urine and feces for nitrogen determination to compare with aliquots of the daily diet. Determinations were made each

TABLE I

Description of subjects

Subject	Age	Height	Weight	Program
No.	*yrs.*	*inches*	*lbs.*	
1	22	70	144	Control
2	29	71	154	Antagonist, 0.5–1.0 Gm./day
3	24	68	161	Antagonist, 1.0–2.0 Gm./day
4	23	72	164	Antagonist, 2.0–4.0 Gm./day

TABLE II

*Diet content of pantothenic acid**

Date	Total pantothenic acid
	mg.
9-30-57	8.615
10-13-57	8.645
10-17-57	7.939
10-25-57	8.399
11- 1-57	8.272
11-11-57	8.068
11-22-57	8.484
12- 2-57	9.357
Average	8.484

* Figures taken from Zook, E. G., MacArthur, M. J., and Toepfer, E. W. Pantothenic acid in foods. U.S.D.A. Agriculture Handbook No. 97, 1956.

week of serum proteins by paper electrophoresis, sedimentation rate, blood counts and urine analysis. Special tests [1] included a Thorn test (eosinopenic response to adrenocorticotropic hormone) (ACTH) and an insulin tolerance test (0.1 unit per Kg. body weight subcutaneously) once each week. Every second week bromsulphalein tests (BSP) and serum bilirubin determinations were made to evaluate hepatic function. Electrocardiograms were taken every second week.

RESULTS

Clinical observation disclosed the following. The control subject, No. 1, had virtually no symptoms throughout the entire study period (Figure 1). On occasions he was slightly irritable. Once he had a cold and for a few days he was drowsy, but his physical examination remained normal. Subject No. 2 who received the smallest dose of omega-methyl pantothenic acid complained on several occasions of a headache which was not severe (Figure 2). He had several colds but his physical state remained normal. Subject No. 3 was well during the first four and one-half weeks of the experimental period (Figure 3). After this he had minor complaints including a mild headache, a cold and, on one occasion, slight nausea, but his physical examination remained normal. After the antagonist was increased to 2 Gm. a day he began to feel ill and was somnolent much of the time. In a few weeks he became irritable and depressed, desiring to be left alone. Later he developed a tremor of the outstretched hands. He had nausea with occasional episodes of vomiting. Administration of pantothenic acid in a dose of 4 Gm. daily was soon followed by alleviation of his symptoms. Subject No. 4, in spite of receiving the largest dose of the anti-vitamin, had no symptoms during the first phase of the study (Figure 4). But when the

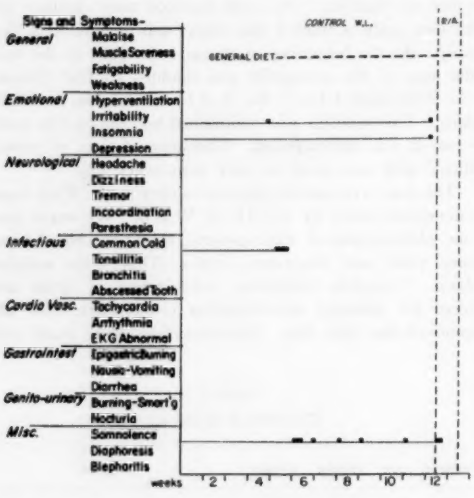

FIG. 1. CLINICAL ASPECT OF PANTOTHENIC ACID DEFICIENCY—CONTROL SUBJECT NO. 1

Subject remained well throughout the study.

[1] By the same methods used in previous studies (1-6).

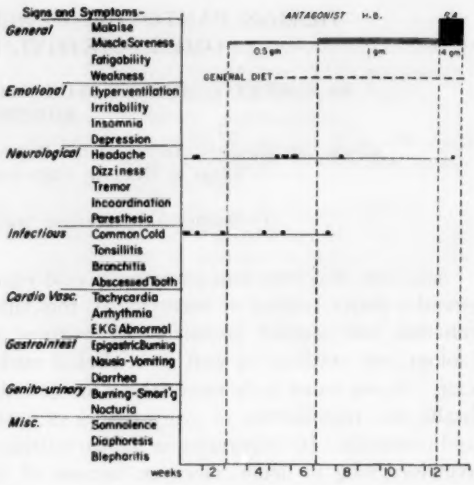

FIG. 2. CLINICAL ASPECT OF PANTOTHENIC ACID DEFICIENCY—SUBJECT NO. 2

Subject No. 2 was given the antagonist, 0.5 Gm. daily, from the third to the seventh week, at which time the dose was 1.0 Gm. daily until the twelfth week. He remained quite well.

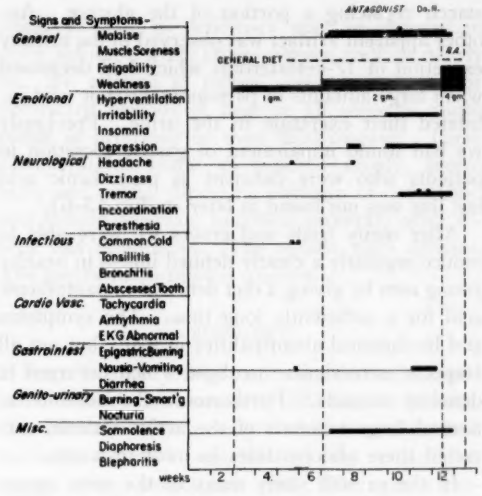

FIG. 3. CLINICAL ASPECT OF PANTOTHENIC ACID DEFICIENCY—SUBJECT NO. 3

Subject No. 3 was given 1 Gm. of the antagonist daily from the third to the seventh weeks, and 2 Gm. daily until the twelfth week. At this time 4 Gm. of pantothenic acid was given daily. He became ill during the seventh week and improved rapidly after pantothenic acid was given.

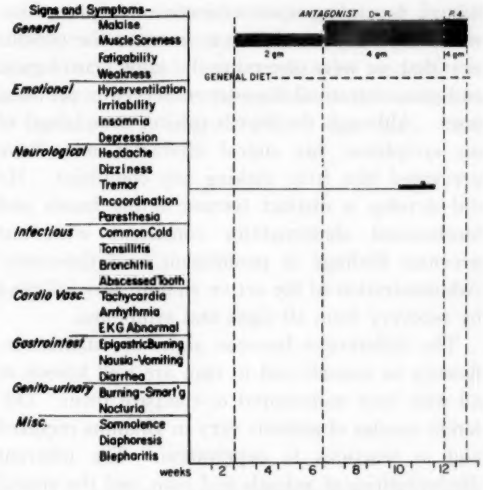

FIG. 4. CLINICAL ASPECT OF PANTOTHENIC ACID DEFICIENCY—SUBJECT NO. 4

Subject No. 4 was given 2 Gm. of the antagonist daily from the third to the seventh weeks and 4 Gm. daily until the twelfth week. He remained well except for a tremor. This subsided spontaneously before pantothenic acid was given, and did not return.

dose was doubled to 4 Gm. daily he developed a tremor of his outstretched hands as the sole abnormality. This disappeared spontaneously a short time before the administration of the vitamin and it did not recur.

None of the subjects developed abnormalities in body weight, temperature, pulse, blood pressure or respiratory rate. The white blood counts, differential counts, hematocrits, urinalyses, electrocardiograms, chest X-rays and the hepatic function studies (BSP and serum bilirubin) remained normal. The erythrocyte sedimentation rate remained normal in Subject No. 1, increased slightly in Subjects No. 2 and 3 and did not change in Subject No. 4. The eosinopenic response to ACTH remained normal in Subjects one and two. In Subject 3 a noticeable decrease in the total eosinophil count developed after he received the antagonist. During the tenth week his eosinopenic response to ACTH became impaired, but recovered and remained normal after pantothenic acid was administered. The man given the largest dose of pantothenic acid antagonist had a sharp

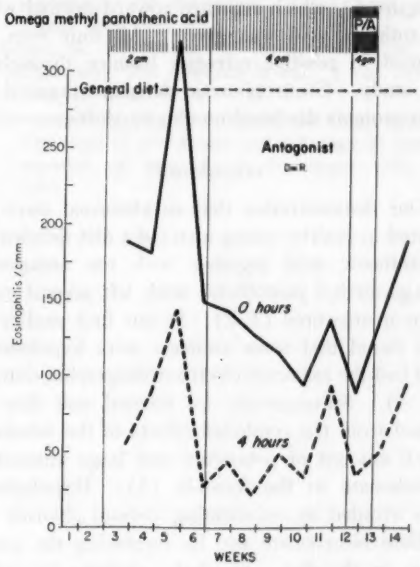

FIG. 5. EOSINOPENIC RESPONSE TO ADRENOCORTICOTROPIC HORMONE (ACTH)

In Subject No. 4, the eosinopenic response was impaired during the eleventh week, but returned to normal when pantothenic acid was given. Note the relative eosinopenia which occurred after the sixth week.

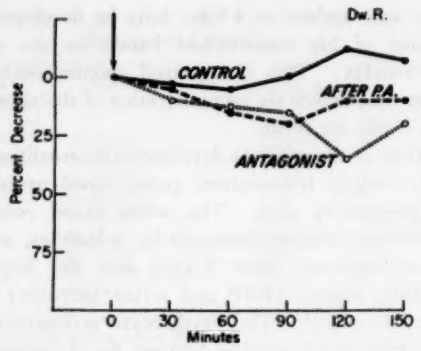

FIG. 6. HYPOGLYCEMIA AFTER INSULIN (0.1 UNIT PER KG. BODY WEIGHT SUBCUTANEOUSLY)

Subject No. 4, who received the largest dose of antagonist, developed increased sensitivity to insulin. This was corrected partially by administration of pantothenic acid.

decrease in his total eosinophil count with gradual development of impaired responsiveness to ACTH (Figure 5). After the administration of pantothenic acid these abnormalities disappeared. He alone developed an exaggerated response to insulin (Figure 6), which returned toward normal when pantothenic acid was given. All four men remained in positive nitrogen balance throughout the study. Electrophoretic determinations of serum proteins disclosed no abnormalities.

DISCUSSION

Our demonstration that an abnormal state appeared in healthy young men fed a diet deficient in pantothenic acid together with the antagonist, omega-methyl pantothenic acid, left several questions unanswered (1, 2). In our first studies we had found that some subjects were hypokalemic and had the expected electrocardiographic changes (3, 4). Subsequently we learned that this resulted from the combined effects of the relatively small amount of potassium and large amount of bicarbonate in the formula (5). Hypokalemia was avoided by substituting sodium chloride for sodium bicarbonate and by increasing the potassium in the diet. Similarly, changes in carbohydrate tolerance apparently had resulted from the composition of the original formula which allowed rapid absorption of glucose resulting in a fast rise and fall of blood sugar. This was corrected by increasing the viscosity of the formula, starch replacing a portion of the glucose. Another apparent artifact was observed in the urinary excretion of 17-ketosteroids which had decreased when large amounts of para-aminobenzoic acid inhibited their excretion in the urine. Previously we had found impairment of gastric secretion in patients who were deficient in pantothenic acid but this was not found in later studies (3–6).

After many trials and errors we were able to induce regularly a clearly defined illness in healthy young men by giving a diet deficient in pantothenic acid for a sufficiently long time. The symptoms and biochemical abnormalities in many but not all respects were similar to those which occurred in deficient animals. Furthermore the administration of large amounts of the active vitamin corrected these abnormalities in most instances.

In the present study many of the same symptoms and biochemical abnormalities occurred in healthy young men fed a balanced nutritious diet and given omega-methyl pantothenic acid. The abnormalities included impairment of the eosinopenic response to ACTH and hypersensitivity to insulin. The slight elevation of the erythrocyte sedimentation rate was considered of doubtful significance. The evaluation of symptoms, although depending upon subjective interpretations, was sufficiently consistent to warrant the conclusion that we were observing the same neurological and gastrointestinal disorders observed in previous tests. Although the fourth subject complained of no symptoms, his stoical character may have prevented him from making any complaint. He did develop a distinct tremor of his hands and biochemical abnormalities consistent with our previous findings in pantothenic acid deficiency. Administration of the active vitamin was followed by recovery from all signs and symptoms.

The differences between signs of vitamin deficiency in animals and in man are well known to all who have endeavored to compare them. Different species of animals vary in vitamins required and in reactions to deprivation. The inherent dissimilarities of animals and man, and the ethical and moral obligations which oblige a physician-investigator to be concerned for the welfare of his patients, add to the complexity of clinical trials. When only a few subjects can be studied, individual differences among subjects add a further complexity. Consequently a partial or in-

complete state of deficiency may be induced in some subjects, while others remain well, or nearly so. To attempt to induce severe symptoms for protracted periods of time in all subjects might result in injurious or even fatal disease. These considerations made us proceed slowly, accept early and partial illness as evidence of deficiency, and restore adequate nutrition promptly after illness was established. The results were definite. The mechanisms remain obscure.

SUMMARY

Four healthy young men were fed a balanced 3,200 calorie diet containing 120 Gm. of protein. One man served as a control while the other three received different amounts of the antagonist, omega-methyl pantothenic acid. The control subject and the man who got the smallest amount of anti-vitamin remained in fairly good health. The man who received an intermediate dose developed gastrointestinal and neurologic symptoms characteristic of pantothenic acid deficiency. The fourth man, who received the largest dose, remained asymptomatic except for the development of tremor.

Biochemical abnormalities were found in the eosinopenic response to adrenocorticotropic hormone (ACTH) of the two men who received the most antagonist, and in the sensitivity to insulin of the man who received 2 to 4 Gm. daily. We conclude that daily administration of 2 to 4 Gm. of omega-methyl pantothenic acid induced clinical and metabolic changes consistent with pantothenic acid deficiency even though a normal diet was fed. This illness was corrected by administration of the active vitamin after we stopped giving the antagonist.

ACKNOWLEDGMENTS

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THE BILIARY EXCRETION OF SULFOBROMOPHTHALEIN SODIUM (BSP) IN THE RAT AS A CONJUGATE OF GLYCINE AND GLUTAMIC ACID*†

By BURTON COMBES‡

(From the Department of Internal Medicine, The University of Texas Southwestern Medical School, Dallas, Texas)

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Sulfobromophthalein sodium (BSP) is a phthalein dye that is removed from blood predominantly by the liver and excreted into the bile (*2*). Removal is impaired in the presence of hepatocellular damage, and BSP retention in blood has proved to be a sensitive index of hepatic dysfunction (3, 4). Although BSP has been used in the clinical evaluation of hepatic disease for over 30 years, the precise mechanisms by which it is handled by the liver remain poorly understood.

Based on a considerable body of evidence (5–14), it is currently believed that hepatic removal of BSP depends upon the simultaneous operation of at least two processes: *1*) uptake of BSP by liver cells in an amount proportional to a given blood level, and *2*) transfer from blood to bile by a rate-limited transfer mechanism. Recently, a chromatographic and chemical analysis of BSP as it appeared in bile was undertaken in our laboratory in order to examine the biochemical mechanisms involved in its excretion. In a preliminary communication (1), it was reported that BSP is excreted as four distinct compounds in the bile of the rat. The major excretory product was demonstrated to be a conjugate of BSP with the amino acids, glycine and glutamic acid. A more complete presentation of these and additional observations forms the basis of the present report.

MATERIALS AND METHODS

The standard BSP used in these studies was the 5 per cent solution sold commercially by Hynan, Westcott and Dunning, Inc., of Baltimore, Md. Chromatographically (*vide infra*), it traveled as a single band with an average R_f of 0.75. S^{35}-labeled BSP was purchased from the Volk Radiochemical Company, Chicago, Ill. The absorption spectra and extinction coefficients of the $BS^{35}P$ and standard BSP were identical. When $BS^{35}P$ was chromatographed, 98.4 per cent migrated with the same R_f as standard BSP (Figure 1). Two compounds accounted for the remaining 1.6 per cent, 1.3 per cent having an R_f of 0.50 and 0.3 per cent an R_f of 0.92.

The common bile ducts of rats (Long Evans, Wistar) were cannulated with fine polyethylene tubing under ether anesthesia. Bile was allowed to drain into small bottles while the rats were gently restrained in special cages. After collection of a control sample of bile, varying doses of BSP were injected and additional bile samples were obtained. The bile samples were treated as follows:

1) Quantitation of BSP in bile. Colorimetric determinations were made on a Beckman DU spectrophotometer, set at 575 mμ, on appropriately diluted bile specimens to which alkali had been added. Complete recovery of BSP was obtained over the range of concentrations, 30 to 350 mg. per cent, encountered in bile in these studies.

2) Chromatography of BSP compounds. Bile samples were applied 25 μl. at a time as a band 5 inches wide on Whatman No. 1 filter paper strips (6 × 21 inches). Each 25 μl. volume was dried with cold air from a hair dryer before the next aliquot was added to the paper. A total of 100 to 200 μl. of bile containing 0.045 to 0.35 mg. of BSP was applied at the origin. The chromatograms were then developed for 16 to 20 hours at 22° C. in a descending system consisting of glacial acetic acid : water : *n*-propyl alcohol (1 : 5 : 10 v/v). After the papers were dried, BSP bands were identified by: *1*) the development of a purplish color when exposed to ammonia vapors, and *2*) their absorption in the ultraviolet range (λ max = 232 mμ).

3) Quantitation of BSP on chromatograms. In some studies, each BSP band was cut out from the chromatograph; the dye was then eluted from the paper into distilled water for 30 minutes and the BSP content of the eluate measured colorimetrically on a Beckman DU spectrophotometer after the addition of alkali. The sum of the optical densities of all the bands accounted for approximately 90 per cent of the BSP originally applied on the chromatogram. For this reason, it was assumed in the following study that the 10 per cent loss of BSP was uniformly distributed among the BSP derivatives. Thus, the percentage of BSP in each band was calculated by

* A preliminary report of these studies has been previously published (1).

† This investigation was supported by research grants from the Dallas Heart Association and from the National Heart Institute of the National Institutes of Health, United States Public Health Service [H-3439].

‡ Established Investigator of the American Heart Association.

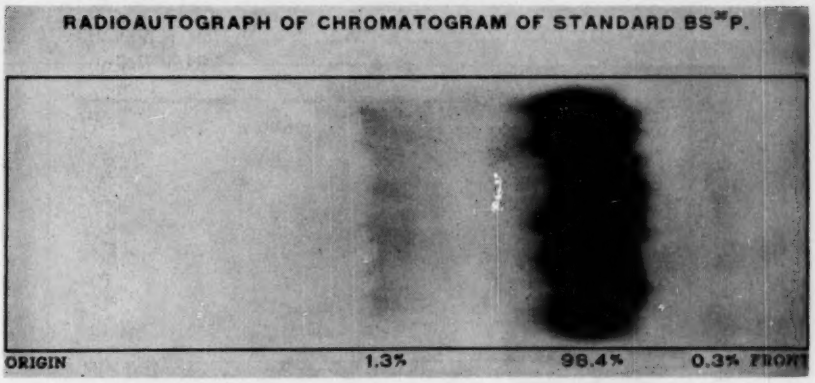

FIG. 1. RADIOAUTOGRAPH OF CHROMATOGRAM OF STANDARD $BS^{35}P$

Ninety-eight and four-tenths per cent of this material migrated with the same R_f (0.75) as standard BSP. One and three-tenths per cent had an R_f of 0.50 and 0.3 per cent an R_f of 0.92.

dividing the optical density derived from that band by the sum of the optical densities of all the bands.

The distribution of BSP in different bands was also determined from segments of the entire chromatograph as follows: Strips 1 cm. wide were cut longitudinally from the chromatograms. The strips were then cut at 1 cm. intervals and the dye contained in each square cm. of paper was measured either colorimetrically or by counting when $BS^{35}P$ was used. For colorimetric analysis, the dye on each square was eluated into 4 ml. of distilled water for 30 minutes and the optical density of the eluate was determined after the addition of 0.1 ml. of 20 per cent potassium hydroxide. When radioactivity was assayed, each square was placed in a counting bottle to which 4 ml. of a 0.4 per cent solution of 2: 5-diphenyloxazole in toluene was added, and the counts were measured in a liquid scintillation counter. A linear counting response was obtained over the range measured in these studies, up to 50,000 counts per minute (cpm).

The optical densities and cpm of the BSP contained in each square were then plotted on the ordinate against the distance of the squares from the origin of the chromatograph in centimeters on the abscissa. Such a plot yielded a series of peaks corresponding to each BSP band. The per cent of BSP in each band was then calculated by dividing the sum of the optical densities or the counts contained in each peak by the sum of the optical densities or counts contained in all peaks. When the per cent distribution of BSP was obtained from colorimetric data, no correction was made for possible differences in the extinction coefficients of the various BSP compounds since values obtained from measurements of radioactivity and color on separate strips from a single chromatogram agreed closely (Table I). Despite the fact that the BSP content of only a segment of the chromatograph was measured, calculation of the per cent of BSP contained in each band from data obtained from individual strips did not differ appreciably from that obtained from measurements of the BSP content of the entire chromatograph (Table II). The quantity of BSP excreted in each band

TABLE I

Comparison of the distribution of sulfobromophthalein sodium (BSP) compounds in bile from radioactivity and color

Rat no.	Band	Counts	Color
		%	%
3	A	59.0	60.7
	B + C	17.7	14.2
	D	23.3	25.1
6	A	52.7	53.7
	B + C	25.3	22.9
	D	22.0	23.4
9	A	79.9	81.7
	B + C	4.7	3.2
	D	15.6	15.1
10	A	71.2	69.1
	B	9.0	8.9
	C	3.6	4.9
	D	16.1	17.1

TABLE II

A comparison of the distribution of BSP in bile calculated from the radioactivity contained in strips and in an entire chromatograph

Rat No. 5	Per cent of BSP contained in		
	Band A	Bands B and C	Band D
Strip 1	67.9	6.5	25.6
Strip 2	64.1	7.5	28.4
Strip 3	67.8	7.4	24.8
Entire chromatograph	66.7	7.2	26.1

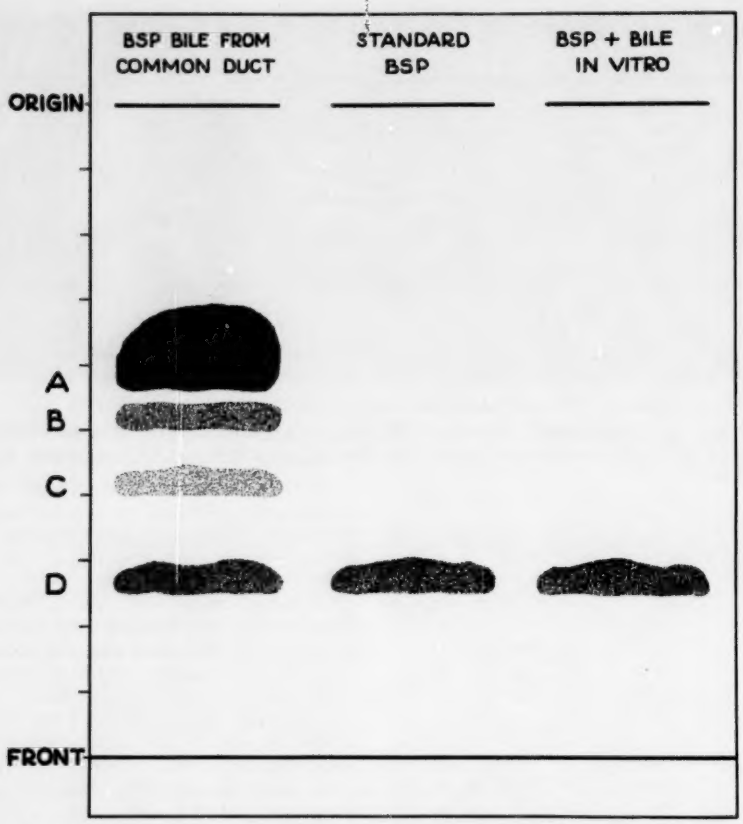

FIG. 2. DRAWING OF A DESCENDING CHROMATOGRAM OF BSP COMPOUNDS ON WHATMAN NO. 1 FILTER PAPER IN GLACIAL ACETIC ACID : WATER : *n*-PROPYL ALCOHOL (1 : 5 : 10, V/V)

was calculated from the values for the per cent of excreted BSP appearing in each band and the total amount of BSP recovered in bile.

RESULTS

1) Chromatography of the biliary excretory products of BSP

Four and occasionally three distinct BSP bands were identified on chromatographs of bile obtained after BSP was administered intravenously. One of these, designated "D", migrated with the same R_f as standard BSP (R_f 0.75), while Bands "A", "B" and "C" moved with average R_f's of 0.44, 0.51 and 0.60, respectively (Figure 2). Separation of Bands B and C varied on different chromatographs. At times an excellent separation was obtained (Figure 2), whereas frequently these bands migrated closely together (Figure 3). When BSP was incubated with control bile for as long as three hours *in vitro* and the mixture chromatographed, only a single band with the same R_f as standard BSP was observed. No evidence of a colorless BSP compound in bile was found after S^{35}-labeled BSP was administered and radioautographs of chromatograms were made (Figure 3).

2) Distribution of BSP compounds in bile (Table III)

Eighty-two to 96 per cent of the injected BSP was recovered in the bile collected in a single tube for two and one-half to three hours following the intravenous administration of varying doses of BSP. An analysis of chromatograms of such bile

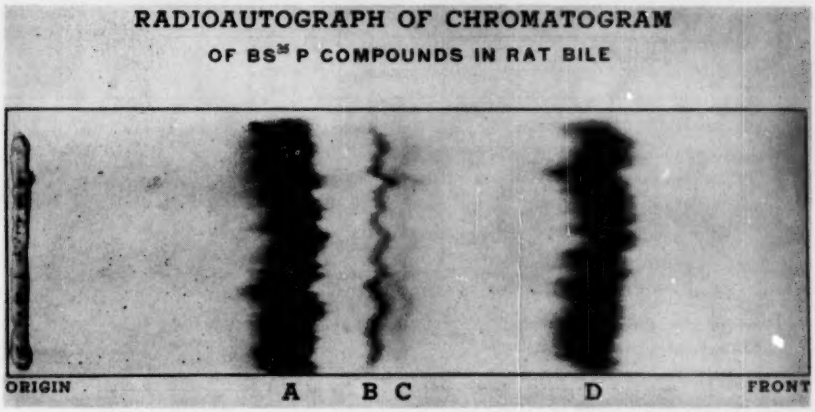

FIG. 3. RADIOAUTOGRAPH OF A CHROMATOGRAM OF BS^{35}P COMPOUNDS IN BILE
Four bands, A, B, C and D, each of which gave a purplish color on exposure to ammonia vapors, were identified. No colorless BSP compounds were detectable.

revealed that Band A contained the major part of the BSP, accounting for over 50 per cent of the dye excreted at the lowest dose administered to as much as 79.7 per cent when 2.62 mg. of BSP per 100 Gm. of body weight was administered. The quantity of BSP appearing in each band rose with increments in dosage, but Band A increased proportionally to a greater extent than Bands B, C and D (Figure 4).

3) Identification of BSP bands

The absorption spectrum of the compounds contained in the different bands is identical with that of standard BSP (Figure 5).

Ninhydrin reacting material is consistently associated with Bands A, B and C. The major compound comprising Band A has been subjected to the following analysis, which indicates that it is a conjugate of BSP with the amino acids, glycine and glutamic acid. When Band A was eluted from paper and subjected to acid hydrolysis (5.9 N HCl for three hours at 15 pounds pressure), it yielded bands corresponding to those of free BSP [1] and two ninhydrin spots. The latter were

[1] When standard BSP is subjected to acid hydrolysis and then chromatographed in acetic acid-water-*n*-propyl alcohol, two BSP bands can be identified. One migrates with the same R_f as standard BSP, the other moves

TABLE III

*Distribution of BSP recovered in rat bile after intravenous injection**

Rat	BSP injected	BSP excreted in bile (mg./100 Gm.)					BSP excreted / BSP injected
		Band A	Band B	Band C	Band D	Total	
no.	*mg./100 Gm.*						
1	0.253	0.119	0.017†		0.088	0.224	0.885
2	0.257	0.111	0.042		0.067	0.220	0.856
3	0.478	0.259	0.078		0.101	0.438	0.924
4	0.482	0.233	0.035		0.172	0.440	0.912
5	0.987	0.596	0.064		0.233	0.893	0.904
6	1.096	0.527	0.254		0.221	1.002	0.914
7‡	1.640	1.170	0.212		0.196	1.578	0.962
8	1.700	1.212	0.102	0.068	0.173	1.555	0.914
9	2.620	1.840	0.109		0.369	2.315	0.883
10	2.650	1.542	0.195	0.078	0.349	2.170	0.818

* Distribution in this table calculated from color in Rats 7 and 8 and from radioactivity in the others.
† The combined values for Bands B and C are listed when these bands could not be sharply separated from each other on the chromatograms for quantitative purposes. In such instances, however, both bands were identified.
‡ Bile collected for 150 minutes. Collection period was 180 minutes in other rats.

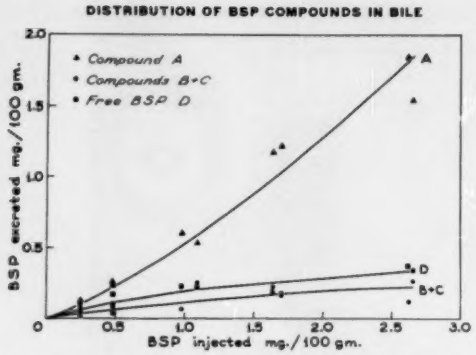

FIG. 4. THE DISTRIBUTION OF BSP COMPOUNDS EXCRETED INTO BILE PLOTTED AS A FUNCTION OF THE QUANTITY OF BSP INJECTED

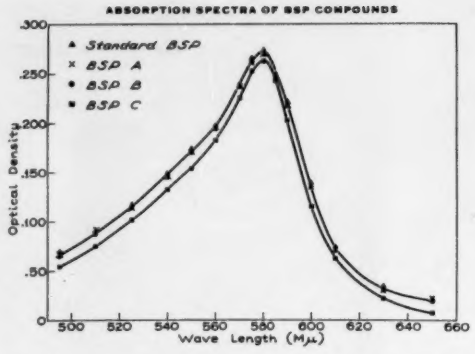

FIG. 5. ABSORPTION SPECTRA OF BSP COMPOUNDS EXCRETED INTO BILE

identified as glycine and glutamic acid by the following: *a*) The ninhydrin spots assumed the positions of glycine and glutamic acid on two-dimensional chromatography in phenol-NH_3 and 80 per cent pyridine; *b*) when the unknown compounds were mixed with known samples of glycine and glutamic acid and chromatographed in the two-dimensional system, the unknown spots were reinforced; only two bands were seen, and these corresponded to the usual location of glycine and glutamic acid in this solvent system; *c*) the dinitrophenyl (DNP) derivatives of the unknown amino acids which were prepared moved with the same R_f's as known DNP-glycine and DNP-glutamic acid in tertiary amyl alcohol-phthalate pH 6.0 buffer (15).

The possibility remained that the glycine and glutamic acid were not conjugated with BSP but appeared in bile either as free amino acids or as a dipeptide that migrated to the same postion as Band A. Indeed, when control bile is chromatographed, ninhydrin reacting material with the same R_f as the BSP band may be identified in some specimens. However, this material can be removed by eluting it from paper and passing it through a Dowex 50–×8 200–400 mesh ion exchange column. Compound A is not affected by this procedure.

A visual comparison of the size of the spots and the intensity of the ninhydrin color developed by glycine and glutamic acid derived from the BSP conjugate with known standards suggests that these amino acids are present in equimolar concentrations and that for each mole of BSP there is a mole of glycine and of glutamic acid. No evidence for free or combined -SH groups was found since negative nitroprusside reactions were obtained on eluates of conjugate A whether previously treated with NaCN or not; and specifically, there was no sign of cysteine, taurine or methionine on amino acid chromatograms.

No evidence of a BSP-glucuronic acid conjugate was found. Thus, chromatograms containing the same volume of control bile and bile containing BSP were developed. Band A and a comparable area from the control bile chromatogram were cut out and their contents were eluted into distilled water. The quantity of hexuronic acid (16) in the eluate of compound A did not differ significantly from that contained in control bile migrating with the same R_f as A, and was much less

faster. These two bands are also found after compound A is hydrolyzed.

TABLE IV

Quantity of glucuronic acid associated with Band A

Rat	Quantity of BSP eluted from Band A	Glucuronic acid	
		Theoretical content if A were a monoglucuronide of BSP	Found in excess of that in control bile
no.	μg.	μg.	μg.
4-14	300	67.1	<0.5
6-21	156	35.0	2.9
7-1	316	70.9	<0.5

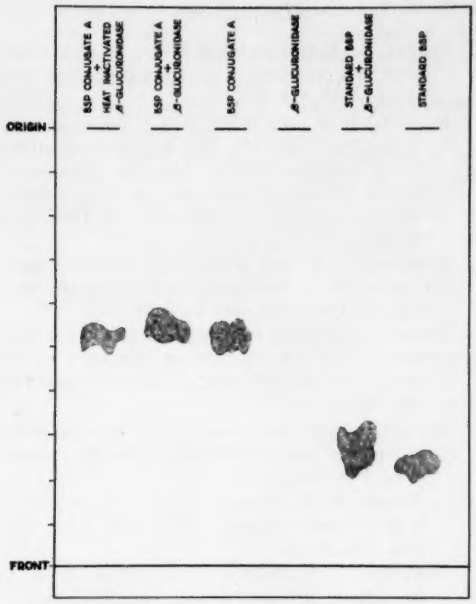

FIG. 6. DRAWING OF A DESCENDING CHROMATOGRAM OF STANDARD BSP AND CONJUGATE A AFTER INCUBATION WITH β-GLUCURONIDASE

No free BSP is liberated from A and the R_f of A is not altered.

than that amount which would have been present if A were a monoglucuronide of BSP (Table IV).

In addition, no free BSP was detected by paper chromatography when compound A was incubated with β-glucuronidase (Worthington) at 37°C., pH 4.9, for one hour, nor was the R_f of A altered (Figure 6). Under similar conditions, the activity of the enzyme was demonstrated by its capacity to liberate phenolphthalein from phenolphthalein glucuronide.

DISCUSSION

The primary purpose of the present report is to describe the nature of the major biliary excretory product of BSP in the rat. This compound, designated BSP A, accounts for up to 80 per cent of the BSP recovered in bile. Ninhydrin reacting material has been found to conform both in shape and position with BSP A on paper chromatograms. This ninhydrin moiety has been demonstrated to be an integral part of the compound since it cannot be removed by passing BSP A through a Dowex 50–×8 ion exchange resin. When subjected to acid hydrolysis, BSP A yields free BSP and two ninhydrin spots. The latter have been identified as the amino acids, glycine and glutamic acid, by means of two-dimensional filter paper chromatography, and this has been confirmed by identifying the dinitrophenyl derivatives of these amino acids. Thus, the major biochemical process concerned with the excretion of BSP into bile is one of conjugation of BSP with glycine and glutamic acid. Ninhydrin reacting material is also associated with BSP B and BSP C, indicating that conjugation with amino acids is also involved in the excretion of these compounds. Studies concerned with the identification of these amino acids are currently in progress.

A preliminary analysis of BSP compounds in human T-tube bile (17) also reveals that the major excretory product is intimately associated with ninhydrin positive material. As in the rat, glycine and glutamic acid are recovered after acid hydrolysis and alanine has been observed in some specimens. These findings in the rat and the preliminary observations in man suggest, therefore, that the BSP test is a measure, at least in part, of amide formation in the liver.

No evidence for a BSP-glucuronic acid conjugate has been found in the present studies in the rat and in man (17). Krebs and Brauer also did not identify a BSP glucuronide compound in rat bile (18). These observations are consistent with the clinical finding that BSP removal by the liver is normal in patients with Gilbert's disease (19), a disorder characterized by impairment of glucuronide conjugation in the liver (20, 21).

The present studies do not exclude the possibility that the BSP molecule itself is altered during excretion by the liver. The absorption spectra of the BSP compounds appearing in rat bile were identical, however, suggesting that the BSP molecule remains intact. Certainly no colorless BSP compounds in bile were found when radioautographs of chromatograms were made after S^{35}-labeled BSP was administered.

Recently, Krebs and Brauer have reported on their separation of BSP products in bile of a variety of animal species by means of chromato-

graphy on alumina columns (18). In the rat, they identified three BSP compounds which they designated "I," "II" and "III." Despite differences in the methods used for separation of BSP compounds in their studies and in the present report, a similarity of the BSP products is apparent. Thus, Compound I was identified as free BSP and is probably the same as BSP D. Compound III accounted for most of the BSP excreted in rat bile. Athough its biochemical nature was not defined, it seems likely that it is identical with BSP A. Compound II may be the same as BSP B or BSP C or conceivably contains a mixture of both B and C.

Evidence has been presented (5–14) which indicates that the hepatic removal of BSP depends upon the simultaneous operation of at least two processes: *1*) uptake by liver cells into a "storage compartment" in an amount proportional to plasma concentration, and *2*) removal from blood by a transfer mechanism characterized by a transfer maximum or Tm. It has been inferred that the Tm is a measure of the movement of BSP from blood to bile (13, 14). The finding of four distinct BSP compounds in bile in the present studies introduces a new complexity in the interpretation of the values for Tm. Henceforth, any analysis of BSP Tm must take into consideration the probability that more than one transport process is operative in the movement of BSP into bile.

SUMMARY

An analysis of sulfobromophthalein sodium (BSP) compounds appearing in the bile of the rat has been undertaken. Usually four and occasionally three BSP bands have been identified by paper chromatography. The major excretory product of BSP in bile, designated BSP A, accounts for up to 80 per cent of the recovered dye. This compound and its hydrolytic products were subjected to both chromatographic and chemical analysis, the results of which indicate that BSP is excreted by the liver of the rat primarily as a conjugate of glycine and glutamic acid.

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HEPATIC METABOLISM OF CHOLESTEROL IN EXPERIMENTAL NEPHROSIS IN RATS *

By RAY H. ROSENMAN, WILLIAM BREALL, SANFORD O. BYERS AND DAVID D. RABIN

(From the Harold Brunn Institute, Mount Zion Hospital and Medical Center, San Francisco, Cal.)

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The nephrotic state induced in rats by injection of rabbit antirat kidney serum (AKS) closely resembles that occurring clinically (1). Our previous studies indicated that the AKS-induced hypercholesteremia in such rats cannot be ascribed to any increase of cholesterol synthesis (2) or intestinal absorption (3), or to any decrease of its intestinal excretion (4). In view of the important role of the liver (5, 6) in the disposition of the plasma cholesterol, we also believed it important to study any possible role of some AKS-induced abnormality of the liver in the genesis of nephrotic hypercholesteremia.

METHODS AND RESULTS

Young adult, male rats (Long-Evans) were used. In preliminary tests the pooled rabbit antirat kidney serum (AKS) (1) used herein was shown to induce significant hypercholesteremia within 24 hours after its intravenous administration. Plasma was variously analyzed for total cholesterol (7), phospholipids (8) and total lipids (9), liver for total cholesterol (10), and bile for bile acid (cholate) (11) concentrations.

A. Effect of nephrosis on hepatic content and hepatic-plasma exchange of cholesterol. Methods: The hepatic cholesterol content was determined in 55 rats at various intervals up to 10 days following injection of 1.0 ml. of AKS. A diet low in fat and sterols was fed during this time. Plasma total cholesterol, and also total lipids in certain instances, were determined at the time of sacrifice. Normal values were established in 20 control rats.

For purposes of comparison, plasma and liver cholesterol also were determined in: *a*) six rats six hours after intravenous injection of 25 mg. of cholesterol, contained in hypercholesteremic rat serum prepared by the hypercholatemic method (12), *b*) in five other rats four days after daily stomach feedings of 100 mg. cholesterol in 3 ml. of olive oil, and *c*) in five additional rats 24 hours after intravenous injection of 100 mg. of Triton® WR-1339.[1]

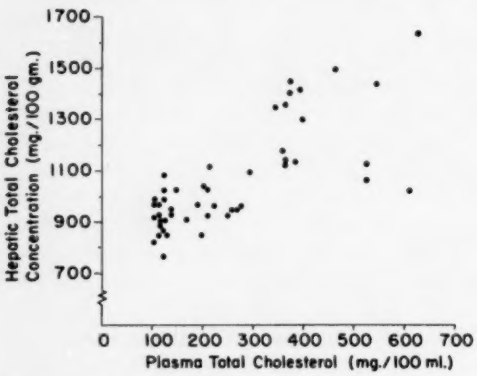

FIG. 1. RELATIONSHIP OF PLASMA AND LIVER CHOLESTEROL IN NEPHROTIC RATS

Results

Hypercholesteremia was induced by AKS 24 hours after its injection (see Table I, Group 2). However, despite the presence of persistent hypercholesteremia, no significant rise of liver cholesterol concentrations occurred until 72 or more hours after AKS injection, and there was no direct relationship between the plasma and liver cholesterol concentrations of these rats (see Figure 1). Although a rise of liver cholesterol occurred after 72 hours following AKS injection (see Table I, Groups 5 to 8), probably indicating the egress from plasma of some of its excess cholesterol content at this time, the plasma and liver cholesterol concentrations remained only poorly correlated (see Figure 1), suggesting that the late rise of liver cholesterol following AKS injection is a function more of time than of the plasma cholesterol concentration.

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[1] Rohm and Haas Co. brand name for *p*-iso-octyl polyoxyethylenephenol polymer.

TABLE I

Hepatic content of cholesterol in nephrotic rats

Group	No. of hours after AKS injection	No. of rats	Average Concentration: Plasma: Total cholesterol	Plasma: Total lipids	Liver: Dry weight	Liver: Total cholesterol: Concentration	Liver: Total cholesterol: Content
			mg./100 ml.		*Gm.*	*mg./100 Gm.*	*mg./organ*
1	0	20	55 (41–68)†	208 (165–233)	2.31 ± 0.14* (1.96–3.16)	893 ± 29.1 (715–1,080)	20.63 ± 0.80 (17.1–24.2)
2	24	15	142 (102–209)		2.03 ± 0.08 (1.54–2.51)	926 ± 15.1 (812–1,030)	18.80 ± 0.96 (16.3–24.8)
3	30	9	116 (101–124)		2.15 ± 0.08 (1.75–2.18)	980 ± 28.3 (767–1,100)	21.07 ± 0.44 (16.7–24.9)
4	48	5	198 (124–250)		2.21 ± 0.27 (1.81–2.52)	984 ± 28.4 (933–1,088)	21.75 ± 2.2 (18.3–24.2)
5	72	8	203 (148–267)	1,392 (992–1,792)	2.25 ± 0.13 (1.81–2.72)	1,021 ± 20.7 (948–1,120)	22.97 ± 1.5 (18.1–30.5)
6	96	10	444 (365–624)	2,135 (1,048–4,055)	2.09 ± 0.07 (1.70–2.40)	1,251 ± 49.2 (1,060–1,640)	26.14 ± 1.2 (21.2–31.7)
7	7 days	5	452 (357–544)		2.20 ± 0.18 (1.75–2.79)	1,342 ± 67.1 (1,134–1,500)	29.52 ± 3.2 (20.0–40.6)
8	10 days	3	370 (364–376)		2.50 (2.23–2.68)	1,329 (1,138–1,420)	33.23 (29.4–31.6)

* Standard error of mean.
† Range of values.

A similar dissociation of plasma and liver cholesterol concentrations was observed (see Table II) in another hypercholesteremic state characterized by the intravascular retention of incoming lipid (13). Thus, a normal content of liver cholesterol was found in rats recently injected with Triton® (13), despite the presence of severe hypercholesteremia (Table II, Group 5). In contrast in the rats injected with (Group 3), or fed excess, cholesterol (Group 4), marked rise of liver cholesterol occurred, the rapidly falling or normal plasma cholesterol levels in such rats indicating the rapid clearance from the plasma and hepatic uptake of such administered excess cholesterol (5).

B. Effect of nephrosis on the hepatic conversion and elimination of cholesterol. Although the previous results indicated that nephrotic hypercholesteremia did not stem from any AKS-induced accumulation of liver cholesterol, it was also believed important to study the effect of nephrosis upon the rate of hepatic elimination of cholesterol, and upon the liver's ability to convert cholesterol

TABLE II

Hepatic cholesterol content in various hypercholesteremic states

Group	Type of rat	No. of rats	Average total cholesterol concentration: Plasma	Liver: Dry weight	Liver: Concentration	Liver: Content
			mg./100 ml.	*Gm.*	*mg./100 Gm.*	*mg./organ*
1	Control	20	55	2.31	893 ± 29.1*	20.6
2	48 hrs. after injection of AKS	5	198	2.21	984 ± 28.4	21.8
3	6 hrs. after injection of hypercholesteremic rat serum	6	154†	2.20	1,285 ± 35.2	28.3
4	4 days after daily gavage of cholesterol and olive oil	5	60	2.61	1,794 ± 150.1	46.8
5	24 hrs. after injection of Triton®	5	440	2.46	913 ± 24.0	22.5

* Standard error of mean.
† Plasma cholesterol averaged 248 mg. per 100 ml. immediately after the injection of hypercholesteremic rat serum.

to cholate, the principal end-product of hepatic metabolism of cholesterol (5).

Methods: 1. Hepatic elimination of exogenously derived cholesterol. The effect of nephrosis on hepatic elimination of cholesterol was studied in rats in which an excess hepatic cholesterol content was induced in two different ways. The first series of 39 rats was fed a diet containing excess cholesterol (3 per cent) and olive oil (3 per cent) for 14 days. Nine rats were sacrificed at this time and the remaining rats transferred to stock ration. Then, 15 of the 30 remaining rats were injected with 1.0 ml. of AKS, the remainder serving as paired controls. Groups of five AKS-injected and five controls were then sacrificed three, seven and 11 days after being transferred to stock ration. In each instance plasma total lipids and total cholesterol and liver total cholesterol were determined. For comparative purpose, similar analyses were done in five normal rats and in five rats injected seven days previously with AKS but fed stock ration.

2. Hepatic elimination of endogenously derived cholesterol. Each of a second series of 70 rats was injected with 3.2 ml. of pooled hypercholesteremic rat serum (HCRS) containing 25 mg. of cholesterol and 60 mg. of phospholipids. Such serum, prepared by feeding cholic acid to otherwise starved rats with ligated bile ducts (12), was found to have only a negligible content of triglycerides. A concomitant injection of 1.0 ml. of AKS was given to 18 of the rats, and groups of six of these latter rats were sacrificed immediately and six and 24 hours later for determination of plasma and liver cholesterol. Five additional rats were sacrificed for similar analyses 48 hours after injection of the HCRS, but were injected with AKS 24 hours after the HCRS administration. The remaining 47 rats served as controls, also being sacrificed at various intervals following injection of HCRS.

3. Hepatic conversion of cholesterol to cholate. The effect of nephrosis upon the hepatic conversion of cholesterol to cholate was studied in a third series of rats. A group of eight rats was fed a diet containing added cholesterol (2 per cent) and olive oil (3 per cent) for 10 days. Three of the rats were then injected with AKS, the remaining five rats serving as their controls. The bile ducts were cannulated (14) and bile collected during the ensuing 24 hours. A second group of 12 rats was injected with 25 mg. of cholesterol contained in 3.2 ml. of the pooled hypercholesteremic rat serum (12), four of the rats having been injected six hours previously with AKS. Bile was collected during a 12 hour interval from these rats. For further control purposes, bile also was collected for 24 hours from a group of eight normal rats

TABLE III

Effect of nephrosis on rate of hepatic elimination of pre-fed dietary cholesterol

Group	No. of days after AKS injection	AKS given*	No. of rats	Average concentration				
				Plasma		Liver		
							Total cholesterol	
				Total lipids	Total cholesterol	Dry weight	Concentration	Content
				mg./100 ml.	*mg./100 ml.*	*Gm.*	*mg./100 Gm.*	*mg./organ*
A. Rats pre-fed fat-cholesterol diet for 14 days								
1	0	–	9	576 (405–785)†	260 (117–363)	4.25 (3.74–4.73)	17,171 (14,120–19,410)	730 (564–865)
2(a)	3	+	5	2,894 (2,110–4,160)	1,226 (1,030–1,597)	3.78 (3.65–3.91)	14,470 (13,100–16,250)	547 (477–636)
(b)		–	5	233 (118–262)	69 (58–95)	4.20 (3.60–5.38)	13,783 (11,940–15,700)	579 (493–643)
3(a)	7	+	5	4,232 (2,807–6,110)	1,857 (1,149–2,494)	3.00 (2.28–3.63)	14,138 (10,730–17,460)	424 (240–588)
(b)		–	5	193 (134–223)	72 (60–92)	3.16 (2.87–3.46)	12,227 (10,670–13,840)	386 (346–422)
4(a)	11	+	5		171 (100–264)	3.12 (2.67–3.88)	3,682 (1,965–5,300)	115 (55–206)
(b)		–	5		52 (47–56)	3.19 (2.74–3.56)	4,619 (2,750–6,900)	147 (91–398)
B. Control rats pre-fed stock ration for 14 days								
5	0	–	5	187 (140–225)	57 (42–68)	2.85 (2.51–3.41)	815 (730–1,000)	23.2 (18.7–25.7)
6	7	+	5	1,518 (763–2,400)	452 (357–549)	2.20 (1.75–2.79)	1,342 (1,134–1,500)	29.5 (19.9–40.6)

* Antikidney serum injections given to Series A rats after 14 days of high lipid diet.
† Range of values.

TABLE IV

Effect of nephrosis on rate of hepatic elimination of excess cholesterol

Group	No. of rats	No. of hours	Average concentration: Plasma: Total cholesterol	Plasma: Phospholipids	Plasma: Total lipids	Liver: Total cholesterol
			mg./100 ml.	*mg./100 ml.*	*mg./100 ml.*	*mg./100 Gm.*
A. Rats injected with hypercholesteremic serum and antikidney serum						
1	6	Immed. postinjection	240 (226–277)*			
2	6	6 hours	156 (139–170)			1,288 (1,300–1,370)
3	6	24 hours	95 (72–129)	154 (135–191)	348 (317–396)	883 (739–985)
4	5†	48 hours	117 (101–124)			858 (767–920)
B. Control rats injected with hypercholesteremic serum						
5	20	Preinjection	55 (42–68)	111 (83–128)	187 (140–225)	893 ± 29.1‡ (714–1,080)
6	8	Immed. postinjection	255 (216–273)	518 (414–708)	787 (678–955)	
7	10	2 hours	212 (191–230)	401 (372–440)	615 (570–640)	1,420 (1,237–1,670)
8	6	6 hours	154 (129–180)			1,285 (1,160–1,400)
9	10	24 hours	61 (47–80)	138 (95–150)	201 (144–230)	871 (678–1,065)
10	13	48 hours	73 (47–87)			855 (714–1,050)

* Range of values.
† These rats injected with antikidney serum 24 hours after injection of hypercholesteremic serum.
‡ S.E. of mean.

fed stock ration. Plasma cholesterol was determined in all rats at the end of the bile collection period and the bile analyzed for its cholate concentration.

Results

As shown in Table III, moderate hypercholesteremia and hyperlipemia and a marked rise of liver cholesterol occurred during the 14 day preliminary period of high lipid feeding (Group 1). Three days after changing to stock ration, plasma lipid concentrations returned to normal in the control rats, and their hepatic cholesterol content diminished somewhat (Group 2b). A further decrease of liver cholesterol occurred by the seventh day (Group 3b); and 11 days (Group 4b) after stopping the diet supplement, the liver cholesterol concentrations had decreased from the initial average level of 17,171 mg. per 100 Gm. to an average of 4,615 mg. per 100 Gm. The injection of AKS into such pre-fed rats induced a severe hyperlipemia and hypercholesteremia (Group 2a) which increased until the seventh day (Group 3a) after AKS injection and then diminished (Group 4a). Despite occurrence of such severe hypercholesteremia, a progressive elimination of the excess liver cholesterol also occurred in these pre-fed, AKS-injected rats which paralleled that in their respective controls, falling to an average of 3,682 mg. per 100 Gm. by 11 days (Group 4a) after transferring to stock diet.

In similar fashion, the development of AKS-induced hypercholesteremia again was not found to interfere with the hepatic elimination of the excess cholesterol contained in the injected hypercholesteremic rat serum. Such serum is essentially free of triglycerides and, as shown in Table IV, its injection also did not induce any rise of plasma triglycerides in the control rats (Groups 6 and 7). Rapid plasma clearance and

hepatic uptake of the injected cholesterol occurred in the control rats (Groups 6 to 10), with complete elimination by the liver of the excess cholesterol within 24 hours after its injection (Group 10). The concomitant injection of AKS was not found to interfere either with rapid plasma clearance and hepatic uptake of the injected cholesterol (compare Groups 2 and 8), or with the subsequent progressive hepatic elimination of the excess deposited cholesterol (compare Groups 3 and 9), despite the occurrence of AKS-induced rise of plasma cholesterol. That the hypercholesteremia present 24 hours after AKS injection (Group 3) was actually induced by the AKS, rather than being due to any AKS-induced delay in plasma clearance of the injected load of cholesterol, is apparent first from the larger ratio of cholesterol to phospholipid in these rats than occurs in rats injected with the hypercholesteremic rat serum (see Groups 6 and 7), and second, from the concomitantly elevated plasma triglyceride concentration which, by calculation of difference between total lipid and the sum of total cholesterol plus phospholipid, averaged 99 mg. per 100 ml. in Group 3 while the corresponding value for Groups 6 and 7 is virtually zero.

Hepatic elimination of cholesterol is normally accomplished primarily by its conversion to cholic acid, which is subsequently excreted via the bile (5). As previously observed (5), therefore, the bile cholate concentration was found to be somewhat increased in the control rats whose hepatic cholesterol content was increased by the prior injection of hypercholesteremic rat serum (see Table V; compare Groups 4 and 5), and markedly increased in the control rats pre-fed excess dietary cholesterol (Group 2). In each instance a comparably increased bile cholate concentration was observed in the similarly treated rats which also had been injected with AKS (Groups 3 and 1, respectively), despite the occurrence of AKS-induced hypercholesteremia in these groups of rats.

C. Possible role of hepatic reticuloendothelial cells (R.E.) in nephrotic hyperlipemia and hypercholesteremia. In view of previous studies (15, 16) of the role of hepatic R.E. cell participation in the plasma egress of exogenously derived cholesterol, it also was believed important to study the possible causal role of this cell system in the genesis of nephrotic hyperlipemia and hypercholesteremia.

Methods: 1. Effect of nephrosis on R.E. cell function. Hepatic R.E. cell function was studied in seven nephrotic rats which had been injected with AKS either 24 hours (four rats) or seven days (three rats) previously. An

TABLE V

Effect of nephrosis on hepatic conversion of cholesterol to cholate

Group	AKS injection	No. of rats	Average concentration: Plasma Total cholesterol	Bile Volume	Bile Cholate Concentration	Bile Cholate Content
			mg./100 ml.	*ml.*	*mg./100 ml.*	*mg./24 hrs.*
A. Rats pre-fed fat and cholesterol for 10 days						
1	+	3	623 (504–743)†	11.7 (9.5–14.5)	563 ± 136.4* (342–890)	65.8 (50.0–85.0)
2	−	5	244 (116–407)	17.3 (13.4–22.0)	392 ± 42.0 (256–545)	67.8 (52.3–103.5)
B. Rats injected with hypercholesteremic rat serum‡						
3	+	4	137 (112–155)		284 ± 16.2 (235–323)	
4	−	8	100 (86–111)		221 ± 6.8 (191–247)	
C. Control rats fed stock ration						
5	−	8	54 (42–69)	14.8 (11.8–17.5)	197 ± 8.7 (152–225)	29.2 (22.1–36.2)

* S.E. of mean.
† Range of values
‡ Bile collected only 12 hours in these rats.

TABLE VI

Effect of India ink injections on plasma lipids of rats concomitantly injected with antikidney serum (AKS)

Type of rat	No. of rats	Average weight	Average plasma concentrations (mg./100 ml.)		
			Total lipids	Total cholesterol	Phospholipids
		Gm.			
Treated*	6	200	644 (446–816)†	198 (132–264)	272 (205–305)
Untreated‡	6	206	680 (404–1,070)	189 (119–237)	286 (187–373)

* AKS plus India ink.
† Range of values.
‡ AKS only.

additional five rats served as controls. During abdominal laparotomy under light ether anesthesia, each rat was rapidly injected via the inferior vena cava with 1.0 ml. of solution containing 10 μc. of $CrP^{32}O_4$ (17) (particle size: 0.2 to 2.0 μ). Four identical samples of 0.034 ml. of blood were then serially pipetted from the previously cut tail at 60 second intervals, each aliquot being spread on a small copper plate, dried, and its activity counted using an end-window Geiger-Müller tube.

Six of a second series of 10 rats also were injected with AKS, and 72 hours later the six nephrotics and four controls were injected with $CrP^{32}O_4$, as above. Blood sampling was done one and five minutes later, and immediately thereafter the hepatic blood supply was clamped, the liver removed, and a thin slice of 40 mg. of liver then washed in saline, dried, and its activity counted. Plasma cholesterol was determined in all rats at the time of sacrifice.

2. Effect of R.E. cell "blockade" on AKS-induced hyperlipemia and hypercholesteremia. The effect of R.E. cell "blockade" on nephrotic rats was then studied. A series of 12 rats was injected with AKS. Six of the rats were also injected intravenously with 1.0 ml. of 20 per cent India ink suspensions twice daily for two days prior to and for two days following AKS injection. All rats then were bled for plasma total lipids, phospholipids and total cholesterol.

Results

Figure 2 shows the rate of plasma clearance of the injected $CrP^{32}O_4$ in the nephrotic and the control rats and indicates that as rapid a plasma egress of radioactivity occurred in both the acute and the chronic nephrotic rats as occurred in the control rats. Plasma total cholesterol averaged 135 mg. per 100 ml. (range, 118 to 167) in the acute nephrotic rats, 523 mg. per 100 ml. (range, 316 to 664) in the more chronic nephrotic animals, and 58 mg. per 100 ml. (range, 48 to 70) in the control group.

Similar rates of plasma clearance of radioactivity again were noted in the second series of rats, the hepatic uptake of radioactivity averaging 234 cpm per mg. of liver (range, 178 to 311) in the nephrotic rats, and 211 cpm per mg. of liver (range, 125 to 260) in the control group.

The repeated injections of the colloidal suspension of India ink did not appear to influence the magnitude of AKS-induced hyperlipemia. Thus, as shown in Table VI, plasma total lipids, cholesterol and phospholipid concentrations were closely comparable in the treated and untreated groups of nephrotic rats.

DISCUSSION

The present results indicate that the rise of plasma cholesterol occurring in AKS-injected rats could not be ascribed to any failure of the liver's ability to eliminate cholesterol by its normal disposal route, the conversion to cholate, which is subsequently excreted in the bile (5). Thus, neither the injection of AKS nor the occurrence

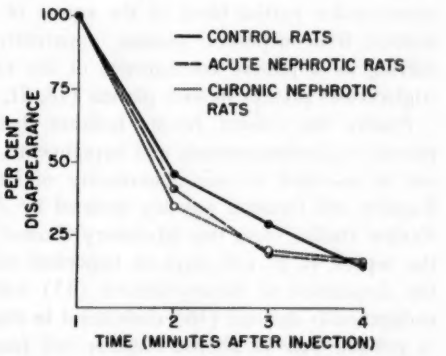

FIG. 2. RATE OF DISAPPEARANCE OF $CrP^{32}O_4$ FROM PLASMA OF NEPHROTIC RATS

of nephrotic hypercholesteremia and hyperlipemia was found to prevent the normal hepatic conversion of cholesterol to cholate and consequent normal rate of hepatic elimination of excess intrahepatic cholesterol. This was found true whether the excess hepatic cholesterol content was derived from an exogenous source, the diet or an endogenous source, hypercholesteremic rat serum obtained from biliary-obstructed rats (12).

The data also indicate that the AKS-induced hypercholesteremia could not be ascribed to any prior or *pari passu* accumulation of cholesterol in the liver. The injection of potent AKS leads to a progressive rise of plasma triglycerides and cholesterol for some 72 to 96 hours after its administration (18). During this phase, the excess cholesterol in the plasma is largely present in a nondiffusible form (19). As a probable consequence, little or no *early* rise of liver cholesterol occurred in the AKS-injected rats, despite their persistent hypercholesteremia, and in contrast to the rapid plasma clearance and rise of liver cholesterol which occurred in rats injected with a load of a diffusible form of excess cholesterol (13, 20). Although a similar mechanism has not been demonstrated in the human disease, a comparable alteration of plasma-liver exchange of cholesterol has been noted in other experimental hypertriglyceridemic states in which associated excess plasma cholesterol is also transported in a nondiffusible form, namely in rats during the first 36 hours after injection of Triton® WR-1339 (13), and in rats continuously infused with triglycerides (21). The present findings are thus consistent with earlier demonstrations (19, 22, 23) of an intravascular partial block to the egress of cholesterol from nephrotic plasma, apparently occurring as a passive consequence of the excess triglycerides present in such plasma (18, 21, 24).

Finally the present results indicate that nephrotic hypercholesteremia and hyperlipemia cannot be ascribed to any abnormality of hepatic Kupffer cell function possibly induced by AKS. Earlier studies from this laboratory showed that the hepatic R. E. cell plays an important role in the disposition of dietary-derived (15) but not endogenously-derived (16) cholesterol in the rat. A possible role of altered Kupffer cell function in the genesis of nephrotic hypercholesteremia was considered because, although of endogenous origin (23), the lipid spectrum of nephrotic plasma resembles an exaggerated alimentary hyperlipemia, characterized as it is by a relatively greater excess of triglycerides (18) and chylomicronemia (24). However, no abnormality of Kupffer cell function, as presently assayed, was found in the nephrotic rats. Moreover, the hypercholesteremia induced by AKS injection was not augmented by the interference with Kupffer cell function (15) consequent to repeated injection of colloidal suspensions of India ink.

SUMMARY

The possible role of any alteration of hepatic parenchymal and Kupffer cell function in the genesis of nephrotic hypercholesteremia was investigated in rats with nephrosis induced by injection of antirat kidney serum. The hypercholesteremia was not found to be ascribable to any accumulation of liver cholesterol, to any failure of hepatic conversion of cholesterol to cholate, or to any abnormality of Kupffer cell function.

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ALDOSTERONE EXCRETION IN VIRILIZING ADRENAL HYPERPLASIA *

By ROBERT M. BLIZZARD, GRANT W. LIDDLE, CLAUDE J. MIGEON AND LAWSON WILKINS

(From the Department of Pediatrics and Division of Endocrinology, The Ohio State University, Columbus, Ohio; Department of Medicine, Vanderbilt University, Nashville, Tenn.; and Department of Pediatrics, Johns Hopkins Hospital, Baltimore, Md.)

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Sodium depletion with concomitant dehydration occurs as a complicating factor in approximately one-third of patients with virilizing adrenal hyperplasia (1). A number of writers have discussed certain differences between the electrolyte disturbance occurring in this syndrome and that of Addison's disease (2–5) and several hypotheses have been offered to explain the sodium loss and potassium retention which occur (1, 4, 6, 7). Although the role of aldosterone is pertinent in many of these hypotheses, the only reported study of aldosterone excretion in patients with virilizing adrenal hyperplasia is that of Prader, Spahr and Neher (6).

In order further to elucidate aldosterone metabolism in this syndrome the present studies were undertaken utilizing subjects both with and without defects of sodium regulation. Accumulated data indicate that patients with virilizing adrenal hyperplasia without associated salt loss excrete normal, or high levels of aldosterone on an *ad lib.* salt intake, and respond with significant increases in aldosterone excretion when placed on limited sodium diets. In contrast, patients with salt loss probably excrete deficient quantities of aldosterone as compared to the normal individual, and are unable to respond with significant increases in aldosterone excretion when sodium intake is limited. Ancillary studies compare the ability of normal infants and adults to excrete aldosterone, both with ingestion of a normal and a sodium deficient diet.

METHODS

Daily 24 hour collections of urine were preserved with refrigeration and thymol. Occasionally two or three day specimens from infants were pooled. In preparation for assay the urine was acidified to pH 1.0 and allowed to stand at room temperature for 24 hours. The "free" steroids were then extracted with dichloromethane. The extracts were washed with aqueous sodium carbonate, dehydrated with sodium sulfate, and evaporated to dryness under a stream of nitrogen. The residues were redissolved in ethanol. These crude extracts were either: *1*) injected into adrenalectomized dogs for assays of aldosterone content by the method of Liddle, Cornfield, Casper and Bartter (8); *2*) subjected to multiple systems of paper chromatography following which the aldosterone fraction was assayed by the same method; or *3*) fractionated and assayed for aldosterone content by the isotope-derivative method of Kliman and Peterson (9). The three methods yielded comparable results when run in parallel on the same urine specimens (Table I). Assays carried out only in adrenalectomized dogs were subject to the limitation of relative lack of sensitivity of this method, *i.e.*, a minimum dose of 1 μg. of aldosterone (per assay animal) was required to induce a distinct response. As a rule an attempt was made to assay each specimen in at least three or four animals. As a result, unless a specimen contained a total of at least 3 or 4 μg. of aldosterone, the responses would be negligible. Under such circumstances it was possible to say only that the amount of aldosterone was "less than" a certain value. In such instances (indicated in the tables and graphs by the symbol $<$ or ↓) the specimen might have contained no aldosterone whatever. This limitation did not apply to the isotope derivative method.

During dietary control periods salt was permitted freely according to each individual's habits. During periods of salt restriction, sodium intake was limited to 9 mEq. or less per day in older patients, and 1 mEq. or less in infants. The sodium free diet for infants consisted of a commercially available low sodium formula,[1] containing 1 mEq. of sodium per L. Although it was necessary to administer sodium chloride to infants with the salt losing form of adrenal hyperplasia in quantities of 45 to 120 mEq. per day in order to maintain life, these patients never were completely hydrated during the collection periods, and were continually bordering on, or were in, adrenal crisis.

Serum sodium and potassium and urinary sodium were

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[1] Lonalac®, Mead Johnson and Co.

determined using a flame photometer with a lithium internal standard. Plasma volume was measured using I^{131} tagged albumin (10). Glomerular filtration rate was calculated using endogenous creatinine clearance as initially applied by Miller and Winkler (11).

RESULTS AND COMMENTS

Most previous investigators studing the physiology of aldosterone have studied only adult patients. It became apparent in the course of the present study that aldosterone excretion is a function of age, and that it was misleading to compare the urinary aldosterone levels of abnormal infants with those of adults, whether normal or abnormal. Table II, therefore, lists the data of aldosterone excretion in seven normal children between the ages of one week and three and one-half years, and in 16 normal adults. Table III similarly lists aldosterone excretion of 16 patients with the non-salt losing form of congenital adrenal hyperplasia, and Table IV, that of 11 patients with the same syndrome but with associated salt loss.

TABLE I

*Comparison of urinary aldosterone assay results by different methods**

Specimen	Dog bioassay		Isotope derivative assay
	Crude extract	Chromatographed extract	
Normal individuals			
1	<2		1
2	4		5
3	12		10
4	3		5
Non-salt losers			
1	<6		3
2	<12		7
3	5	8	
4	6	5	
5	16	8	
6	7	11	
7	11	7	
8	10	4	
9	9	19	
Salt losers			
1	2		1
2	8		4
3	9		5
4	2		4
5	4		2
6	<1		2
7	<1	<1	
8	2	3	
9	4	6	
10	<1	<1	
11	<4	<4	

* All results are expressed in micrograms per 24 hours.

Since in normal infants the urinary excretion of aldosterone and the response to sodium deprivation is apparently less than in older children and adults, it is necessary to consider the findings in the patients with the salt losing and non-salt losing types of virilizing adrenal hyperplasia in relation to their age groups.

I. Aldosterone excretion in normal control subjects (Table II)

A. Adults. Fourteen of 16 adults studied during an *ad lib.* salt intake excreted between 3 and 8 μg. of aldosterone per 24 hours. One patient (C.Y.) excreted 1 μg., and one patient (A.R.) 9 μg. We have, therefore, regarded the normal adult excretion value under these circumstances to be 3 to 8 μg. per 24 hours. With sodium restriction to 9 mEq. or less per day, aldosterone excretion increased to between 20 and 66 μg. per day.

B. Infants. Four infants, less than two months of age, were studied and three were subjected to a low sodium diet. Aldosterone excretion during the control period was probably less than the 3 to 8

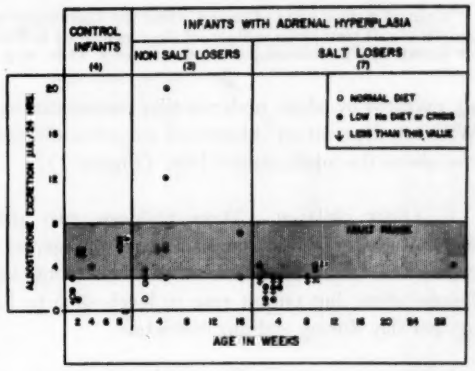

FIG. 1. ALDOSTERONE EXCRETION IN NORMAL INFANTS AND INFANTS WITH VIRILIZING ADRENAL HYPERPLASIA

The shaded area represents the normal adult range of aldosterone excretion when adults are on *ad lib.* salt intakes. Since both on a low salt diet and during a spontaneous adrenal crisis the intravascular volume is decreased, and since this is a known stimulus to aldosterone production (18), these two stress situations are assumed to be comparable (see key, ●).

TABLE II

*Aldosterone excretion in normal adults (A1-16), normal infants (B17-20) and normal older children (C21-23)**

Patient	Age	Rx	Aldosterone (μg./24 hours) Day of low sodium diet: Control	1	2	3	4	5	6	Aldosterone method	Comment
A-1 E.L.	18 years	0	4	2	6	9	19	15		D.A.-C.E.	
2 W.D.	18 years	0	5	6	8	12	20	40	66	D.A.-C.E.	
3 C.Y.	18 years	0	1	10	9	16	28	37	38	D.A.-C.E.	
4 A.R.	19 years	0	9	12	10	16	28	38	32	D.A.-C.E.	
5 E.M.	19 years	0	6	6	8	12	16	23	22	D.A.-C.E.	
6 V.B.	19 years	0	7	6	10	19	27	32	39	D.A.-C.E.	
7 R.F.	20 years	0	5	4	4	5	11	13	47	D.A.-C.E.	
8 H.W.	22 years	0	4	4	6	5	9	19	21	D.A.-C.E.	
9 R.K.	24 years	0	4	7	7	14	16	36	25	D.A.-C.E.	
10 J.J.	25 years	0	3	3	5	8	13	20	34	D.A.-C.E.	
11 W.M.	25 years	0	6	6	8	12	19	27	34	D.A.-C.E.	
12 D.D.	27 years	0	8	9	11	20	30	29	34	D.A.-C.E.	
13 M.B.	32 years	0	6	7	12	15	23	33	52	D.A.-C.E.	
14 Y.N.	47 years	0	8	10	15	20	32	35	30	D.A.-C.E.	
15 I.C.	48 years	0	4	4	7	10	12	13	20	D.A.-C.E.	
16 H.M.	73 years	0	3	3	4	9	15	7	20	D.A.-C.E.	
B-17 D.O.	1 week	0	<2			3	2			D.A.-C.E.	Urinary Na, 3 mEq. 4th day Serum electrolytes, normal
18 D.F.	2 weeks	0	<6				1	<6		D.A.-C.E.	Urinary Na, 3 mEq. 5th day Body wt., 2,130–1,970 Gm. 5th day
19 C.P.	4 weeks	0	4							D.A.-C.E.	
20 C.C.	8 weeks	0	<6				6	6	6	D.A.-C.E.	Urinary Na, 2.0 mEq. 5th day Serum electrolytes, normal
C-21 J.E.	2.5 yrs.	0	2				6		7	D.A.-C.E.	
							4			I.D.	
22 L.C.	3 years	0	<2					4		D.A.-C.E.	
			1					6		I.D.	
23 L.N.	3.5 yrs.	0	3				12	10		D.A.-C.E.	
			2					12		I.D.	

* Control values were obtained while the individuals were maintained on an unrestricted sodium diet. During the periods of salt restriction sodium intake was limited to 9 mEq. or less per day for Groups A and C and less than 1 mEq. for Group B. The following symbols are used: D.A., dog assay; C.E., crude extract; I.D., isotope derivative assay.

μg. excreted by adults under similar circumstances. With salt deprivation aldosterone excretion did not rise above the adult control level (Figure 1).

C. Older children. Three children, two and one-half to three and one-half years of age, excreted less than adult levels of aldosterone on regular diets, but output rose to levels of 6 to 12 μg. per day during sodium restriction.

II. Aldosterone excretion in infants with non-salt losing, virilizing adrenal hyperplasia (Table III, A)

Three children (D.K., B.S., L.S.), 16 weeks of age or younger, were subjected to a low salt diet. At this time cortisone therapy had not been initiated. In two of the three patients aldosterone excretion significantly increased during salt deprivation (Figure 1). One infant (B.S.) six weeks of age, excreted 20 μg. of aldosterone on the fifth day of sodium deprivation, responding quantitatively as would an adult. None of the infants manifested clinical dehydration or serum electrolyte alterations.

III. Aldosterone excretion in infants with salt losing, virilizing adrenal hyperplasia (Table IV)

Seven infants who had not been previously treated, and who were admitted to the hospital in, or bordering on, salt losing crisis were studied. Salt retaining steroids and cortisone were not used therapeutically prior to or during the initial study. One patient (S.M.) was subsequently restudied while receiving cortisone. Although salt administration in limited amounts was necessary to maintain life, the urine collections were made

while the patients were still dehydrated, and often hyponatremic and hyperkalemic.

Nineteen determinations for aldosterone were performed on 15 urines of these seven patients who were 30 weeks or less of age. In two instances the aldosterone values were 4 μg. per 24 hours. The remainder of the determinations were less than this value. In some cases it was only possible to state that the urine contained less than the designated value because of the limitations of the dog assay method (Figure 1). It is possible that there may have been no aldosterone in these samples.

IV. Aldosterone excretion in older children and adults with non-salt losing, virilizing adrenal hyperplasia (Table III)

Aldosterone excretion was measured in 13 patients between four and three-fourths and 32 years of age who had never had symptoms of salt loss. Of 19 determinations in this age group obtained while these patients were receiving normal sodium intakes, 14 were above the adult range of 3 to 8 μg. per day and five were within this range (Table III, Figure 2). Four of the patients were receiving cortisone therapy at the time the studies were

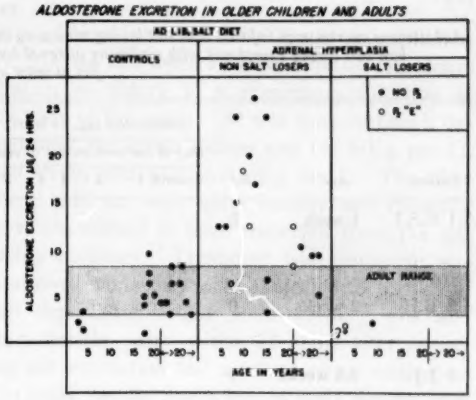

FIG. 2. ALDOSTERONE EXCRETION OF 19 CONTROL SUBJECTS BETWEEN TWO AND ONE-HALF AND 73 YEARS OF AGE, 13 INDIVIDUALS HAVING NON-SALT LOSING, VIRILIZING ADRENAL HYPERPLASIA BETWEEN FOUR AND THREE-FOURTHS AND 32 YEARS OF AGE, AND THREE OLDER CHILDREN BETWEEN ONE AND THREE-FOURTHS AND NINE YEARS OF AGE HAVING *PREVIOUSLY* HAD SALT LOSING SYMPTOMS WITH VIRILIZING ADRENAL HYPERPLASIA

None of the last group had required salt retaining steroids for at least one year. All subjects were on an unrestricted sodium intake. The shaded area represents the normal adult range of aldosterone excretion when adults are on an *ad lib.* salt intake.

TABLE III

*Aldosterone excretion in infants (A1-3), older children and adults (B4-16) with non-salt losing, virilizing adrenal hyperplasia**

Patient	Age	Rx	Aldosterone (μg./24 hours) Day of low sodium diet: Control	1	2	3	4	5	Aldosterone method
A-1 D.K.	1 week	0	1			4	3	3	I.D.
								<6	D.A.-C.E.
2 B.S.	5 weeks	0	3, <6				12	20	D.A.-C.E.
3 L.S.	16 weeks	0	3					7	I.D.
								<12	D.A.-C.E.
B-4 C.W.	4 years	0	12						D.A.-C.E.
5 C.A.	6 years	0	12						D.A.-C.E.
6 H.T.	7 years	"E"	6						D.A.-C.E.
7 A.B.	8 years	0	24, 15				38		D.A.-C.E.
8 J.D.	10 years	"E"	18						D.A.-C.E.
9 C.L.	11 years	"E"	12			8	6		D.A.-C.E.
10 T.T.	11 years	0	20						D.A.-C.E.
11 D.B.	13 years	0	26, 17				41		D.A.-C.E.
12 C.L.	16 years	0	6, 3			12	10		D.A.-C.E.
13 N.K.	16 years	0	24						D.A.-C.E.
14 E.H.	25 years	"E"	12, 8				18	7	D.A.-C.E.
15 J.L.	25 years	0	10						D.A.-C.E.
16 M.H.	32 years	0	9, 9, 5					36	D.A.-C.E.

* During the periods of salt restriction sodium intake was limited to less than 1 mEq. per day for Group A and less than 9 mEq. per day for Group B. The following symbols are used: "E", cortisone (25 to 37.5 mg. per day); D.A., dog assay; C.E., crude extract; I.D. isotope derivative assay.

TABLE IV

*Aldosterone excretion in infants with salt losing, virilizing adrenal hyperplasia (A 1-7) and older children who had previously had salt losing symptoms with virilizing adrenal hyperplasia, but who had required no salt retaining steroid for at least one year (B 8-11)**

Patient	Age	Rx	Aldosterone (μg./24 hours) Day of low sodium diet or crisis: Control	1	2	3	4	5	Spontaneous crisis	Aldosterone method	Comment
A-1 E.S.†	1 week	0							4, <3	D.A.-C.E.	Serum Na, 134–139 mEq. Serum K, 6.3–8.2 mEq.
									<4, <1	D.A.-Ch.E.	Severe dehydration
2 B.H.	2 weeks	0							<1, <2	D.A.-C.E.	Serum Na, 122–131 mEq. Serum K, 5.4–6.0 mEq. Moderate dehydration
3 J.J.	2.5 weeks	0							<2	D.A.-C.E.	Serum Na, 131–143 mEq. Serum K, 9.8 mEq. Moderate dehydration
4 K.S.	2.5 weeks	0							1, <3	D.A.-C.E.	Serum Na, 130–138 mEq. Serum K, 6.5 mEq.
									<1	D.A.-Ch.E.	Severe dehydration
5 D.H.	4 weeks	0							2, <3	D.A.-C.E.	Serum Na, 143–159 mEq. Serum K, 5.9–6.3 mEq.
									2	D.A.-Ch.E.	Severe dehydration
6 S.M.	8 weeks	0							<3, (3×)	D.A.-C.E.	Serum Na, 117–126 mEq. Serum K, 6.5–7.6 mEq. Moderate dehydration
	9 weeks	15 mg. E/os q.d.							<4, (2×)	D.A.-C.E.	Serum Na, 135 mEq. Serum K, 8.6 mEq. Moderate dehydration
7 J.A.	30 weeks	0							4	D.A.-C.E.	Serum Na, 130 mEq. Serum K, 6.2 mEq. Moderate dehydration
B-8 S.W.	1 9/12 years	"E"	1		1‡					D.A.-C.E.	Control day / Crisis day‡
					<1					D.A.-Ch.E.	Serum Na, 138 mEq. / 124 mEq. Serum K, 5.7 mEq. / 7.2 mEq. Body wt., 9,930 Gm. / 9,300 mg. Urinary Na, 166 mEq. / 22 mEq. Dehydration—none / Severe
1 E.S.†	2 1/2 years	"E"							1	D.A.-C.E.	Serum Na—? / 146 mEq. Serum K—? / 6.0 mEq. Dehydration—none / Severe
9 G.M.	2 10/12 years	"E"					6			D.A.-C.E.	Serum Na, 134 mEq. / 118 mEq. Serum K, 5.3 mEq. / 6.4 MEq.
								<1‡		D.A.-Ch.E.	Urinary Na, 96 / 10 Dehydration—none / Severe
10 B.O.	3 years	"E"	<2					2‡		D.A.-C.E.	Serum Na, 153 mEq. / 138 mEq. Serum K, 6.7 mEq. / 5.6 mEq. Body wt., 14.1 Kg. / 13.0 Kg. Urinary Na—? / 22 mEq. Dehydration—none / Moderate Not in cl. crisis
11 L.W.	9 years	0	2 1	<1 2	4 2	2 4	8 4	9 5‡		D.A.-C.E. I.D.	Serum Na, 141 mEq. / 140 mEq. Serum K, 4.8 mEq. / 5.2 mEq. See Figure 4A

* During the period of salt restriction (Group B) sodium intake was limited to less than 9 mEq. per day. The following symbols are used: "E", cortisone (15 to 37.5 mg. per day); D.A., dog assay; C.E., crude extract; Ch.E., chromatographed extract; I.D., isotope derivative assay,‡ crisis day;† same patient.

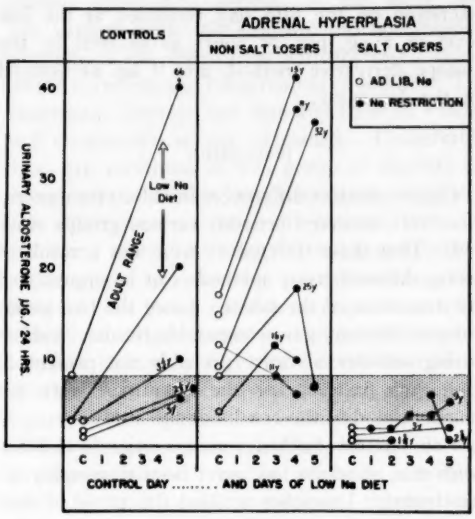

FIG. 3. THE RESPONSE IN ALDOSTERONE EXCRETION TO A 9 MEQ. RESTRICTED SODIUM DIET BY NORMAL INDIVIDUALS, PATIENTS WITH VIRILIZING ADRENAL HYPERPLASIA WITHOUT ASSOCIATED SALT LOSING SYMPTOMS, AND PATIENTS WITH SALT LOSING FORM OF VIRILIZING ADRENAL HYPERPLASIA

The symbol *y* indicates age in years. The shaded area represents the normal adult range of aldosterone excretion when adults are on an *ad lib.* salt intake.

made. This had no apparent suppressive or enhancing effect on aldosterone excretion.

Six of these patients were subjected to a low sodium diet for four or five days. With one exception this caused a significant increase of aldosterone excretion (Figure 3). As in normal individuals, sodium was conserved by diminution of urinary excretion, serum electrolytes remained stable, and body weight diminished gradually (Figure 4B).

V. Aldosterone excretion in children with adrenal hyperplasia who previously had salt losing symptoms (Table IV)

Studies were carried out on four children, one and three-fourths to three years of age who had previously had salt losing symptoms requiring desoxycorticosterone acetate (DCA) pellet implanatation or 9 alpha-fluoro-hydrocortisone (Figure 3). At the time of the study they had required no salt retaining steroid for at least one year but were maintained on suppressive doses of cortisone with a liberal intake of salt. One of these children (E.S.) had developed an adrenal crisis with dehydration secondary to a respiratory infection at the time of the study. At this time, although dehydrated, the serum sodium was 143 mEq. per L., and serum potassium, 6.0 mEq. per L. The other three children were quite healthy and clinically were considered to have recovered from the salt losing tendency. Treatment with cortisone was continued while they were subjected to periods of salt deprivation. One child (S.W.), one and three-fourths years, within 72 hours after initiating salt restriction had a fall of serum sodium to 124 mEq. per L., and a loss of body weight from 9,900 to 9,300 Gm. The aldosterone excretion remained less than 1 µg. per 24 hours, and urinary sodium excretion remained 22 mEq. per 24 hours

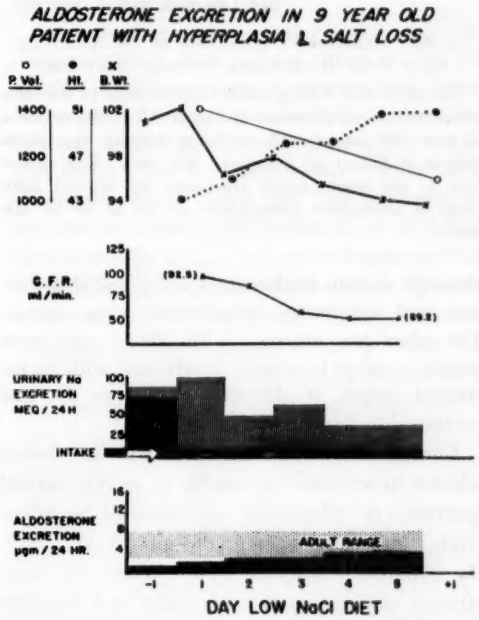

FIG. 4A. THE EFFECT OF A 9 MEQ. SODIUM DIET ON PLASMA VOLUME (P. VOL.), HEMATOCRIT (HT.), BODY WEIGHT (B. WT.), GLOMERULAR FILTRATION RATE (G.F.R.) AND URINARY SODIUM AND ALDOSTERONE EXCRETION IN A NINE YEAR OLD PATIENT WITH VIRILIZING ADRENAL HYPERPLASIA HAVING A HISTORY OF ASSOCIATED SALT LOSS

Salt retaining steroids, however, had not been required for several years. The shaded area in the lower graph represents the normal adult range of aldosterone excretion when adults are on an *ad lib.* salt intake.

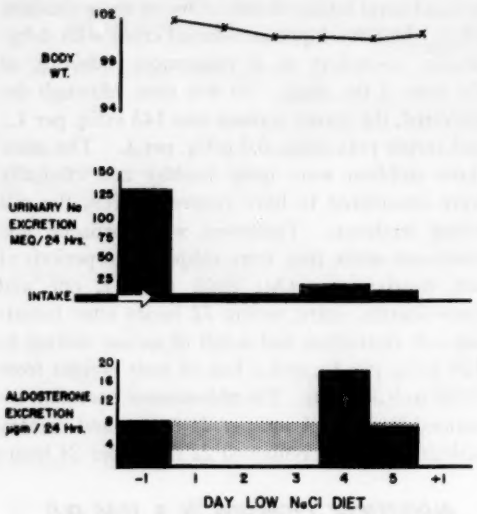

FIG. 4B. ALDOSTERONE EXCRETION IN AN ADULT PATIENT WITH HYPERPLASIA WITHOUT SALT LOSS

The effect of a 9 mEq. sodium diet on some of the same parameters as diagrammed in Figure 4A is shown for a 32 year old patient with virilizing adrenal hyperplasia without a history of associated salt loss. The shaded area in the lower graph represents the normal adult range of aldosterone when adults are on an *ad lib.* salt intake.

although sodium intake was 1 mEq. and dehydration and circulatory impairment were severe. The other two infants (G.M., B.O.) also were unable to adapt to sodium deprivation with an increased output of aldosterone (Figure 3), and continued to lose sodium in the urine.

Further evidence that patients with salt losing adrenal hyperplasia are unable to secrete normal quantities of aldosterone was obtained by intensively studing a nine year old girl (L.W.) with the syndrome (Figure 4A). While on unrestricted sodium intake, this child had required no salt retaining steroid for several years. On a restricted sodium diet for five days her body weight decreased from 102 to 94 pounds. Simultaneously the hematocrit rose from 43 to 51, the plasma volume fell from 1,395 to 1,135 ml., and the glomerular filtration rate fell from 92.5 to 59.2 ml. per minute. On the fifth day sodium excretion was still 43 mEq. per 24 hours indicating inability to conserve sodium. Aldosterone excretion on the fifth day remained at the low level of 5 µg. per 24 hours as assayed by the isotope derivative method, and 9 µg. as assayed by the dog assay.

DISCUSSION

Certain distinct differences in aldosterone excretion were observed between various groups studied. That these differences were not a result of using different assay methods can be appreciated by inspection of the tables. Since the two variations of bioassay gave comparable results, "sodium losing substances" were probably not present in the crude extracts or the quantities were too small to modify the results of the study.

Comparison of aldosterone excretion in children with that of adults has never been thoroughly investigated. Luetscher studied the urine of four normal children, four to eight years of age, maintained on a liberal sodium intake, and determined the aldosterone content to be approximately one-half that of normal adults (12). Our limited studies presented in this report suggest that aldosterone excretion in childhood is proportionate to the child's size, as is true for corticosteroid excretion (13).

Aldosterone excretion of patients with virilizing adrenal hyperplasia also has never been extensively studied. The only report has been that of Prader and co-workers (6) who reported aldosterone assays on urine of six patients with virilizing adrenal hyperplasia, two without salt loss and four with this complication. One of the two individuals without salt loss excreted an increased quantity of aldosterone and the other a normal amount. Two of the four patients with salt loss excreted normal quantities of aldosterone and two excreted increased quantities. Our data confirm those presented by Prader and associates for individuals having non-salt losing, virilizing adrenal hyperplasia, but differ from theirs for individuals having the salt losing form of the disease. In our investigation none of the 11 patients with salt loss excreted elevated amounts of aldosterone. Our data suggest that aldosterone excretion by this group may, in fact, be low in comparison to normal or non-salt losing, virilizing

adrenal hyperplasia patients of the same age. The patients with salt loss also did not respond normally to salt deprivation with increased aldosterone excretion and conservation of sodium. The discrepancy between our data and those of Prader and co-workers is not explained. Conceivably there are variations in this group of patients although all our studies have consistently suggested low aldosterone excretion in patients with the salt losing type of virilizing adrenal hyperplasia.

One might speculate that the failure of sodium deprived, salt losing patients to respond normally with increased aldosterone excretion is that hydrocortisone deficiency may prevent this normal response. That hydrocortisone does not explain the failure of these patients to increase their urinary aldosterone and conserve sodium during salt deprivation is indicated by the following considerations: *1*) "Salt losers" on cortisone therapy were no better able to respond than those not receiving cortisone (Table IV) during salt deprivation, and *2*) patients with hydrocortisone deficiency secondary to panhypopituitarism have been shown to retain their capacity to increase aldosterone excretion during sodium deprivation (14, 15).

A second consideration as to the etiology of the low levels of aldosterone excretion is the decreased glomerular filtration rate observed in L.W., and undoubtedly present in the dehydrated patients. It is unlikely that change in glomerular filtration rate accounts entirely for the low urinary levels of aldosterone as patients with other diseases in which glomerular filtration rate may be low excrete increased quantities of aldosterone (12, 16, 17). Normal production of aldosterone, therefore, probably does not occur in these patients, even in the presence of decreased intravascular volume, which is one of the primary stimuli for aldosterone production (18). Consequently an inherent assumption in the present study is that urinary aldosterone levels reflect the rate of secretion of this hormone.

Whether some factor in addition to the limited capacity to secrete aldosterone might play a role in the salt losing form of virilizing adrenal hyperplasia remains obscure. Differences in the sodium losing tendency of these patients and of patients with Addison's disease were noted by Crigler, Silverman and Wilkins (4). They concluded that the steroids secreted by the "abnormal adrenals" of the former group caused sodium loss either by antagonizing the action of "sodium retaining" hormone or because of a specific "sodium losing" factor. This hypothesis was based on the observation that considerably greater amounts of desoxycorticosterone were necessary to control the salt loss in untreated than in cortisone treated patients, and that corticotropin administration caused sodium diuresis. In addition, Wilkins, Klein and Lewis (3), in 1950, reported one patient who had salt losing symptomatology only secondary to adrenocorticotropic hormone (ACTH) administration. Many workers subsequently have unsuccessfully attempted to isolate a specific "sodium diuretic" steroid from the urine of patients with the salt losing type of virilizing adrenal hyperplasia.

Recently Bongiovanni and Eberlein (7) have demonstrated a more severe defect in the production of hydrocortisone in patients with the salt losing type of virilizing adrenal hyperplasia than in those without this complication. They suggest that the salt loss is related to the severity of the enzymatic defect. Since both hydrocortisone and aldosterone require hydroxylation of 21-desoxyhydrocortisone at C_{21}, and since Bongiovanni and Eberlein (19), and Jailer, Gold, Vande Wiele and Lieberman (20) have shown that patients with virilizing adrenal hyperplasia, except those with the hypertensive type, have varying defects of hydroxylation at C_{21} it might be expected that the "salt losers" would be unable to excrete normal quantities of aldosterone. Our data would suggest that such is the case.

Deficiency of aldosterone in this group is not unexpected as Lewis, Klein and Wilkins (21) demonstrated the absence of the zona glomerulosa in the salt losing type of virilizing adrenal hyperplasia. Giroud, Stachenko and Piletta (22) have shown that this is the only zone of the beef adrenal that is capable of producing aldosterone, and Ayres Garrod, Tait and Tait (23) have confirmed these findings in human adrenal.

Our data suggest also that some patients with non-salt losing adrenal hyperplasia excrete greater than normal amounts of aldosterone when on un-

restricted salt intake. If confirmed in subsequent studies this would support the hypothesis that there is a "sodium losing" factor produced by the adrenals of most patients with virilizing adrenal hyperplasia, but that compensation occurs if aldosterone production can be increased. Prader and associates (6) have been proponents of this theory.

SUMMARY

Aldosterone excretion has been measured in seven normal children, age one week to three and one-half years, and in 16 adults maintained on a normal and low salt diet. The data indicate that normal children do not excrete adult levels of aldosterone but are probably capable of responding to a low salt intake with moderately increased excretion of aldosterone.

Individuals with virilizing adrenal hyperplasia, but without salt loss, excrete normal or high levels of aldosterone, and are able to respond normally to a low sodium diet with increased aldosterone excretion and sodium conservation.

In contrast, data are presented suggesting that patients with virilizing adrenal hyperplasia with associated salt loss are unable to excrete normal quantities of aldosterone even when they are dehydrated and have diminished intravascular volume. It is therefore concluded that impaired ability to synthesize aldosterone probably contributes to the disturbed electrolyte balance characteristic of this syndrome.

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NORMAL CONCENTRATIONS OF SOME TRACE METALS IN HUMAN URINE: CHANGES PRODUCED BY ETHYLENEDIAMINETETRAACETATE *

By H. MITCHELL PERRY, JR.† AND ELIZABETH F. PERRY

(From the Hypertension Division, Department of Internal Medicine, Washington University School of Medicine and Barnes Hospital, Saint Louis, Mo.)

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Interest in the biologic role of trace elements prompted an investigation of their renal excretion. Preliminary qualitative analysis revealed that except for cobalt the entire first transition series of metals, namely, titanium, vanadium, chromium, manganese, iron, nickel and copper, were detectable in most human urines. In addition the heavier transition metals, molybdenum and silver, as well as the related metals, zinc, cadmium, tin and lead, were ordinarily present. A method was evolved by which the urinary concentrations of such metals could be quantitatively estimated. The procedure included initial chemical concentration followed by spectrographic analysis. Contamination was minimized by careful preparation of equipment and reagents. Since extraneous iron and copper were particularly difficult to avoid and since these two metals had frequently been studied by other techniques, no attempt was made to measure them spectrographically. The exclusion of significant positive contamination, the demonstration that the low renal excretion of cobalt permitted its use as an internal standard, and the determination of standard working curves and recovery data for each metal constituted the control data for the assay procedure. Estimations were less satisfactory for chromium and titanium than for the remaining nine metals.

Collection and assay of 24 hour urines were performed in a standard manner. Such urines from normal persons served to indicate both the mean level and the variability of excretion for the nine trace metals which could be satisfactorily estimated. Daily differences in the renal excretion of metal as well as the effects produced by extremes of urinary volume and pH were determined for 24 hour urines from one normal individual. A brief preliminary study was made of the metallic spectrum in single specimens of urine from hospitalized African natives. Finally, metal concentration was measured in consecutive 24 hour urines from hypercholesterolemic patients before, during and after the parenteral administration of the chelating agent, disodium calcium ethylenediaminetetraacetate, which had been observed to lower the level of cholesterol in human plasma (1).

METHODS

Preliminary qualitative urinalysis. In order to select the metals to be assayed, 40 single urine specimens from different subjects were analyzed qualitatively. Half of the samples were obtained from normal individuals and half from hospitalized patients with atherosclerosis and hypertension. The urines were evaporated to approximately one-twentieth of their original volume, after which most of the organic material was oxidized with hydrogen peroxide. The residues were then dried, mixed with an approximately equal amount of graphite, and burned to completion in a direct current arc. The photographed spectra were examined, several levels of intensity being provided for each sample by use of a rotating step-sector disc.

As was expected, magnesium and calcium were always found, strontium was present in all except seven samples, but barium was not identified. Aluminum was also invariably found. Boron was excreted by one patient, a 63 year old hypertensive female whose urine contained boron both before and after she was treated with hydralazine. Every element of the first transition series from titanium to copper was identified in more than one sample, although cobalt was present only twice and then in barely detectable traces. Molybdenum and silver were the only heavier transition elements found. Zinc, cadmium, tin and lead were the only additional nontransition metals detected aside from the alkali metals.

Chemical concentration. A homogeneous 200 ml. aliquot of each 24 hour urine sample was measured into a 400 ml. glass Berzelius beaker containing a glass stirring

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† Established Investigator, American Heart Association.

rod and glass beads. All glassware used in the analysis was rendered *metal-free* by special cleaning to be described. Approximately 10 ml. of *redistilled concentrated nitric acid* was added to the beaker which was then covered with a watch glass. The mixture was boiled cautiously for several hours until its volume was reduced to about 10 ml. An additional 20 ml. of the acid was carefully added, and the mixture was again boiled to a volume of 10 ml. If the solution remained dark, 10 to 20 ml. more of the acid was added to complete the oxidation of organic matter. Except for urines with considerable amounts of protein, a total of 50 ml. of *redistilled concentrated nitric acid* was adequate to produce 10 ml. of a clear amber solution. A maximum of 50 ml. of *redistilled concentrated hydrochloric acid* was then cautiously added, in several portions, to the hot nitrated residue in order to reduce the excess nitric acid and to convert the nitrates to chlorides. After each addition the resulting solution was once again carefully boiled down to a volume of 10 ml. When reduction was complete a very pale yellow color replaced the original red or orange color. The cool solution was diluted to 150 ml. with *redistilled metal-free water*. An internal standard consisting of 1.00 ml. of *0.00200 per cent aqueous cobalt* was added. As a carrier for precipitataion and as a matrix for ignition, 5.0 ml. of *0.5 per cent aqueous aluminum oxide* was also added.

The remainder of the chemical concentration was as described by Mitchell and Scott under the heading "Precipitation by 8-hydroxyquinoline + tannic acid + thionalide" (2). Briefly, 10 ml. of *5 per cent 8-hydroxyquinoline in 2 normal distilled acetic acid* was added, and the pH of the solution was adjusted to 1.90 ± 0.05 by the dropwise addition of approximately 8 normal *purified ammonium hydroxide*. After an hour, 30 ml. of *purified 2 normal ammonium acetate* was added, bringing the pH to approximately 4.8. Next, 2.0 ml. of *10 per cent aqueous tannic acid* and 2.0 ml. of *1 per cent thionalide in glacial acetic acid* were added. The pH then was readjusted to 5.10 ± 0.05 by the addition of as much as 30 ml. of approximately normal *purified ammonium hydroxide*, after which the mixture was allowed to stand for 24 to 48 hours. Finally the precipitate was collected on 11 cm. Whatman No. 54 filter paper and washed several times with *redistilled metal-free water*.

Spectrographic analysis. The filter paper containing the washed precipitate was packed in a covered *metal-free* platinum crucible and placed in a muffle furnace. The temperature of the furnace was raised to just above 100° C. and kept there until the paper and the precipitate were nearly dry. The temperature was then increased to 420 to 425° C. and kept there for no more than six hours, after which time the filter paper was virtually gone. Because of the volatility of cadmium, great care was taken to keep the time and temperature of heating to a minimum. After cooling, the residue was ground in a *metal-free* agate mortar with an approximately equal portion of *graphite powder*. Approximately 10 mg. of the ash and graphite sample was placed in the crater of a *sample electrode* which was made the cathode in an 8 ampere direct current arc. The electrode gap was made 5 mm. and the region of the arc 1 mm. above the cathode was focused on the collimeter of an ARL 1.5 M. grating spectograph with a slit width of 50 μ. During the entire 25 seconds of burning, the spectrum from 2,250 to 4,500 Å was photographed on Eastman SA No. 1 film, each roll of which was calibrated by the two-step method. The film was developed for three minutes in D-19 developer, immersed for 15 seconds in 3 per cent acetic acid, fixed for seven minutes in Kodak Acid Fixer, and washed for 10 minutes in running water, all at 20° C. The percentage transmissions for each of the lines indicated in Table I and of the chromium line at 4,254.3 Å were determined on a densitometer, and after correction for background the values obtained were converted to intensity ratios with respect to the 3,334.1 Å cobalt line. In order to increase accuracy, each sample of ash and graphite was burned in duplicate.

Equipment and reagents. Metal-free equipment was prepared by rendering it visibly clean with detergent and allowing it to stand overnight in equal parts of concentrated nitric and sulfuric acids. Each piece was then successively rinsed a half-dozen times in tap water, in distilled water, and in the *redistilled metal-free water* described below, after which it was allowed to dry in air under a Parafilm® cover. All glass and polyethylene equipment which came in contact with the original urine, with aliquots at any stage in the analytic procedure, or with *metal-free* reagents was so treated to render it *metal-free*. All reagents and urines were stored in polyethylene containers. All distilling apparatus was made entirely of glass.

Redistilled metal-free water was prepared by putting doubly distilled water through an ion exchange resin. All water used in the preparation of reagents was purified in this manner and had a conductivity of less than 1.0 micromho.

Redistilled concentrated nitric acid and *redistilled concentrated hydrochloric acid* were prepared by twice distilling concentrated analytic reagent grade acid. Approximately 15 and 9 normal acids were obtained, respectively. Enough nitric acid to perform a large number of analyses was prepared and pooled so that it would not prove a source of short term variation.

0.00200 per cent aqueous cobalt was prepared with reagent grade cobaltous chloride. The final solution contained 20 mg. of cobalt per L. so that 1.00 ml. in 200 ml. of urine yielded a concentration of 100 parts of cobalt per billion parts of urine.

Purified ammonium hydroxide with a concentration approximating 8 normal was prepared by distilling ammonia from reagent grade ammonium hydroxide and collecting it in water.

0.5 per cent aqueous purified aluminum oxide was prepared by precipitating aluminum hydroxide from an aqueous solution of reagent grade (chromium-free) aluminum chloride with *purified ammonium hydroxide*. The precipitate was washed and then dissolved in warm *redistilled concentrated hydrochloric acid*. The resulting solution

TABLE I

*Experimentally determined intensity ratios from which standard working curves were constructed**

	Mn	Pb	Mo	V	Cd	Sn	Zn	Ti	Ag	Ni
					Lines used (in Angstrom units)					
	2,593.7	2,833.1	3,170.3	3,185.4	3,261.1	3,262.3	3,282.3	3,372.8	3,382.9	3,423.7
					Minimum amounts of metals added to artificial urine (in μg. /L.)					
	19.5	6.88	2.73	1.09	7.45	8.05	117	5.72	0.820	7.20
					Experimentally determined intensity ratios					
1 X	0.34, 0.37	0.80, 0.94	0.45, 0.46	0.27, 0.27	0.24, 0.26	0.49, 0.52	0.45, 0.49	0.39, 0.42	1.55, 1.63	0.45, 0.54
Min.	0.36, 0.37	0.83, 0.84	0.47, 0.51	0.29	0.24, 0.25	0.67, 0.73	0.45, 0.48	0.48, 0.53	1.54, 1.74	0.39, 0.43
Amt.	0.35	(0.78, 1.21)	0.45			(0.61, 0.96)	0.47, 0.52	(0.29, 0.81)	1.53, 1.62	(0.39, 0.97)
2 X	0.67, 0.76	1.05, 1.13	0.69, 0.76	0.53, 0.56	0.47, 0.49	1.06, 1.08	0.91, 0.93	0.90, 0.90	2.98, 2.72	0.88, 0.91
Min.	0.74, 0.75	1.16, 1.17	0.84, 0.93	0.52, 0.54	0.49, 0.53	1.20, 1.36	0.73, 0.89	0.86, 0.72	2.80, 3.12	0.75, 0.76
Amt.	(0.71, 0.97)	(0.83, 1.11)	(0.97, 1.27)	(0.19, 1.57)	(0.45, 0.56)	(0.76, 1.14)	0.76, 0.89	(0.93, 1.64)	2.96, 3.18	0.84, 0.89
4 X	1.42, 1.46	1.58, 1.43	1.43, 1.59	1.04, 1.24	0.96, 1.00	2.32, 2.55	1.41, 1.61	1.80, 2.16	6.31, 5.50	1.64, 1.79
Min.	1.30, 1.44	1.51, 1.53	1.44, 1.74	1.20, 1.27	0.99, 1.08	2.24, 2.45	1.52, 1.77	(0.64, 4.80)	5.85, 6.37	1.56, 1.93
Amt.	(1.48, 1.86)	(1.18, 1.58)	(1.52, 1.96)	(0.99, 1.77)	(1.09, 1.48)	(2.26, 3.06)	1.39, 1.64	(1.72, 3.29)	6.10	1.60, 1.78
8 X	2.64, 2.94	1.80, 2.06	2.66, 2.80	2.01, 2.28	1.77, 1.87	3.50, 4.24	3.11, 3.70	3.89		2.58, 3.06
Min.	2.44, 2.58	1.87, 2.24	2.72, 2.84	2.17, 2.29	1.84, 1.97	4.12, 4.90	2.52, 2.96	4.37		3.69, 4.01
Amt.	(2.73, 3.53)	(2.02, 4.16)	(2.76, 3.82)	(2.10, 2.89)	(1.60, 2.01)	4.48	(2.88, 4.18)			3.42
16 X	5.10	(2.71, 4.90)	4.98, 5.24	3.93, 4.25	4.19, 4.80		4.96, 4.63			
Min.		(2.12, 3.85)	5.19	4.08, 4.20	4.66, 4.70		4.42			
Amt.		2.92		4.18	4.32		4.87			
32 X		3.62		7.90, 8.44						
Min.		3.37								
Amt.										

* Intensity ratios were experimentally determined for six concentrations of trace metals. Three aliquots of each concentration were assayed in duplicate. All of the experimentally determined intensity ratios are tabulated unless the metal line transmitted less than 5 or more than 75 per cent of the densitometer light. Single values and places where both values are absent therefore indicate the frequency of metal lines which were too dense or too light for accurate assay. When pairs of values failed to agree within 20 per cent, both were enclosed in parentheses.

was extracted thrice with reagent grade diethyl ether, heated to remove excess ether, and suitably diluted.

Distilled glacial acetic acid was prepared by distilling reagent grade glacial acetic acid (Mallinckrodt).

5 per cent 8-hydroxyquinoline in 2 normal distilled acetic acid, 10 per cent aqueous tannic acid, and *1 per cent thionalide in distilled glacial acetic acid* were prepared weekly with reagent grade 8-hydroxyquinoline (Mallinckrodt), reagent grade tannic acid (Eimer and Amend 0.000 per cent heavy metal as lead), and high purity thionalide (Merck), respectively.

Purified 2 normal ammonium acetate was prepared by titrating *distilled glacial acetic acid* with *purified ammonium hydroxide* to yield a solution of pH 7.

Graphite powder was Ultra Purity Graphite Powder UCT-2-200 obtained from United Carbon Products Company, Inc., Bay City, Mich.

Counter electrodes were Preformed Ultra Purity Graphite Counter Electrodes No. 100 U made by United Carbon Products Company.

Sample electrodes were made from one-fourth by 12 inch U-2 Hi Purity Graphite Rods obtained from United Carbon Products Company. An ellipsoidal depression 2 mm. deep and 6 mm. in diameter was cut in one end of a 40 mm. section of the rod.

Control data for assay. In order to be certain that the reagents and equipment introduced no positive contamination, an artificial urine without organic components was prepared. *Redistilled metal-free water* was made 0.10 molar with respect to sodium chloride, 0.050 molar with respect to potassium chloride, and 0.010 molar with respect to both magnesium and calcium chlorides. To four separate 200 ml. aliquots of the artificial urine, 50 ml. of *redistilled concentrated nitric acid* and then 50 ml. of *redistilled concentrated hydrochloric acid* were added. Thereafter each of the four samples was assayed for manganese, lead, molybdenum, vanadium, cadmium, tin, zinc, titanium, silver, nickel and chromium, in the manner described. Using the lines indicated in Table I for the first 10 metals and the chromium line at 4,254.3 Å, no detectable trace of any of these metals was found.

In order to confirm the suitability of cobalt as an internal standard, single urine samples of more than 200 ml. from 12 apparently healthy individuals were collected directly into *metal-free* containers. Twelve hospital patients who were receiving no drugs and who had a variety of diseases including hypertension, atherosclerosis, cirrhosis, nephrosis, diabetes, idiopathic hypercholesterolemia and lead poisoning furnished similar specimens. An additional 12 specimens were obtained from patients with the same diseases during a period of intravenous disodium calcium ethylenediaminetetraacetate therapy. Except that no internal standard was added, all 36 specimens were assayed in the manner described. No trace of the cobalt line at 3,334.1 Å was found.

In order to obtain standard working curves from which the metal concentrations in actual urines could be determined, known quantities of 11 metals were added to the previously mentioned artificial urine. Under the head-

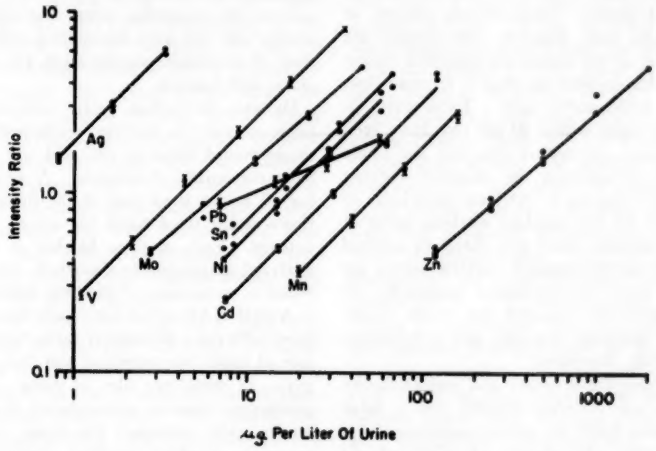

FIG. 1. STANDARD WORKING CURVES FOR CONVERTING *INTENSITY RATIO* TO *METAL CONCENTRATION*

The curves were plotted from the data given in Table I. Each point is the average of a pair of values which agreed to within 20 per cent. Zinc, nickel and molybdenum points are indicated by solid dots. Manganese, lead and vanadium points are indicated by crosses. Cadmium, tin and silver points are indicated by open circles. No curve is given for titanium since the data were considered inadequate.

TABLE II

Reproducibility of metal assay in human urine

	Mn	Pb	Mo	V	Cd	Sn	Zn	Ti	Ag	Ni
	Mean concentrations of metal in pool of normal urine on duplicate analyses (μg./L.)									
	24.0	9.81	21.6	2.70	10.7	9.17	120	10.2	0.87	14.8
	Metal added to urine pool (μg./L.)									
	39.0	13.8	5.46	2.18	14.9	8.05	117	11.4	1.64	7.20
	Mean for 10 replicate assays after the above amounts of metal were added to urine pool (μg./L.)									
	58.0	25.5	26.8	4.51	26.2	18.2	241	20.6	2.56	22.5
*S.E.M.**	*1.7*	*1.0*	*1.0*	*0.41*	*0.51*	*0.77*	*4.3*	1.7	*0.044*	*0.61*

* Standard errors of the means.

ing, *minimum amounts of metals added*, Table I indicates 10 of the metals and the concentration of each that was used. These metals were added as the chlorides except for vanadyl sulfate, ammonium molybdate, silver nitrate and lead acetate. In addition, 5.73 μg. of chromium per L. was added; however, the assay for chromium was unsatisfactory and no quantitative data are given for this metal. Three identical 200 ml. aliquots of the artificial urine with the added metals were assayed in duplicate as described. The experimentally determined pairs of intensity ratios and their corresponding metal concentrations are given in Table I. Additional artificial urines with the previously mentioned concentrations of alkali metals and alkaline earths were made two, four, eight, 16, and 32 times as concentrated with respect to each of the 11 transition and related metals. Three 200 ml. aliquots of each of these solutions were assayed. The results are given in Table I. All of the values are tabulated except when the metal line transmitted less than 5 or more than 75 per cent of the densitometer light. Those pairs of values which did not agree within 20 per cent have been enclosed in parentheses, and neither they nor the single values were used in constructing the standard working curves, which comprise Figure 1. At least eight pairs of values were available for the standard working curve of each metal, except titanium which was therefore omitted from the figure. All of the standard working curves approximated straight lines over the ranges examined. All also closely approached the expected unit slope, except lead which deviated markedly and zinc and molybdenum which deviated slightly therefrom.

In order to determine the accuracy and reproducibility of the assay, urine was collected directly into a large metal-free polyethylene bottle by several apparently normal laboratory workers. An aliquot was analyzed and found to contain no detectable cobalt. The mean concentration of each of the 10 metals, including titanium, was determined by the method outlined. The results of the initial duplicate assay are listed at the top of Table II. Below are tabulated the known amounts of each metal which were then added to the pool. After the additions, 10 separate 200 ml. aliquots were assayed. The experimentally determined mean metal concentration after the additions and the standard error of that mean are indicated for each metal. The coefficients of variation for the 10 replicate assays were calculated from these data and were found to vary from 5 to 6 per cent for cadmium, zinc and silver, to 25 to 30 per cent for vanadium and titanium.

Collection and assay of 24 hour urines. With precautions to avoid contamination, 24 hour urine samples were collected directly into wide-mouthed, *metal-free* gallon jars to which no preservative had been added. Immediately after additions, the jars were carefully covered with Parafilm®. No funnels were used. As soon as 24 hour collections had been completed, homogeneous 600 ml. aliquots were transferred to *metal-free* polyethylene containers for storage at 2° C. until analysis. The volume of the remaining urine was measured, its specific gravity and pH were determined, and a quantitative estimate of its protein concentration was made with Esbach's picric acid reagent.

Despite distillation, nitric acid remained the most likely source of positive contamination. Therefore a single pooled batch of the acid was used for all urines from the normal Americans. A second batch was used for all urines from four of the hypercholesterolemic patients and a third batch for urines from the remaining patients. Each of these batches of acid was made and analyzed as previously described; no 50 ml. aliquot was found to contain any of the nine elements considered here.

A total of 144 urines were each assayed for nine metals; thus, 1,296 pairs of intensity ratios were determined. Each pair of ratios was averaged and then converted to micrograms of metal per liter of urine. In 88 instances the conversion required extrapolation since the averaged intensity ratio exceeded the range covered by the appropriate standard working curve. Extrapolation assumed continued linearity of the standard working curves and was only permitted when the per cent transmission of the lines of both the internal standard and the element in question were between 5 and 75 per cent. Extrapolated values cannot be considered absolute, but they suggest relative concentrations. No extrapolation was needed for vanadium, cadmium or tin; and it was only necessary once for manganese and once for silver. One of

the normal Americans and seven of the hospitalized Africans had more urinary nickel, and four of each group had more urinary molybdenum, than could be assayed without extrapolation. Although the normal zinc excretion was confined to the range covered by the standard working curve, the zincuria during the intravenous administration of disodium calcium ethylenediaminetetraacetate required extrapolation for 42 of 50 urines. It was the lead assay which was the most disturbing, however. The two unpaired intensity ratios for 220 μg. lead per L. (Table I) suggest that the standard working curve remained linear at least to that concentration. Two urines from normal Americans and 26 urines from seven of the eight hypercholesterolemic patients required extrapolation beyond this extension of the standard working curve for lead.

Although quantitative data on the excretion of titanium and chromium are not cited, intensity ratios for the 3,372.8 Å titanium line and for the 4,254.3 Å chromium line suggested the approximate concentrations of these two metals. The intensity ratio was also determined for the cadmium line at 2,288.0 Å because it was about five times as sensitive as the line at 3,261.1 Å, but reproducibility was poor.

MATERIAL

One 24-hour urine specimen was collected by each of 24 apparently normal individuals. Ten were Negroes, of whom six were female. Among the 14 non-Negroes, four were female; two of the four were in the last trimester of pregnancy. The ages ranged from four to 69 years with two being in the first, two in the second, nine in the third, six in the fourth, one in the fifth, two in the sixth, and two in the seventh decade of life. To minimize environmental differences the sexagenarians were the grandfathers of the two youngest children, both of whom were boys. Nine individuals were laboratory workers, seven were other professional people, five were students, and three were housewives. Each continued to eat his regular diet and to continue his usual daily activities during the collection period. All were working and none was hypertensive. Each urine was examined for proteinuria, but none was found. The collected urines were individually assayed for metals as described.

Six consecutive 24 hour urine specimens were collected by one normal 24 year old white male physician while he was eating his regular diet and working in the laboratory. He collected six more specimens under similar conditions at various other times. The interval between the first and last of these collections exceeded two years. The same individual collected 12 additional 24 hour urine specimens under conditions designed to produce acidic, basic, concentrated and dilute urines. Four consecutive 24 hour urine specimens were collected during a four day period when 12 Gm. of ammonium chloride was ingested daily Similar collections were made during a four day period when 12 Gm. of sodium bicarbonate was ingested daily. A pair of consecutive collections was obtained during a two day period of restricted fluid intake. Similar collections were made during a two day period when fluids were forced. In none of the 24 specimens collected by this individual was protein demonstrated.

In Usumbura, Ruanda-Urundi, Africa, single urine specimens of more than 100 ml. were collected directly into wide-mouthed, *metal-free*, 6 ounce polyethylene bottles. The 12 subjects were all hospitalized normotensive natives between the ages of 17 and 34 years. Half were male. None was more than superficially exposed to *Western civilization*. The major diagnoses were idiopathic epilepsy and unexplained anemia in three each, parasitic infestation in two, and bacterial pneumonia, mitral disease, tuberculous peritonitis, and bronchial asthma in one each. The urines were assayed as described except that 100 rather than 200 ml. aliquots were used. Since the full amount of internal standard was added, the concentration of cobalt was relatively high. An assay was considered unsatisfactory, and therefore repeated, unless the transmission of the internal standard line was less than 40 rather than 75 per cent.

Two to four consecutive 24 hour urine specimens were collected directly into *metal-free* gallon bottles by eight patients with hypercholesterolemia. Over an approximately three hour period during the next day, seven of the patients received 1.0 Gm. of intravenous disodium calcium ethylenediaminetetraacetate ($Na_2CaEDTA$) dissolved in 500 ml. of 5 per cent glucose in water. On the subsequent three to nine days, each of these seven patients received 3.0 Gm. of $Na_2CaEDTA$ given in the same manner. The eighth patient received 3.0 Gm. of $Na_2CaEDTA$ for two days and 4.0 Gm. on the third day as described (3). During the entire period of chelate administration and in four cases for one or two days thereafter, consecutive 24 hour urine specimens continued to be collected in *metal-free* bottles. The patients ranged in age from 21 to 63 years. Three were women and one was a Negro. None was on a diet, nor was any receiving other drugs. The primary diagnosis of five was atherosclerosis with objective evidence of involvement of the coronary arteries; the three other major diagnoses were lead poisoning, pseudobulbar palsy and nephrosis. None had hypertension. Only the nephrotic patient had proteinuria; his urine contained from 1.6 to 3.2 Gm. of protein per L. (3). Before chelate therapy the concentration of cholesterol was measured in the plasma of each patient; it ranged from 265 to 460 mg. per 100 ml. At least one subsequent level was obtained for each patient while he was receiving $Na_2CaEDTA$. For three patients levels were followed daily before, during and after $Na_2CaEDTA$ administration. The collected urines were assayed as described.

RESULTS

Part I of Table III lists the mean concentrations of nine metals in the 24 hour urines of 24 normal adults. The standard error of the mean, the range of concentrations, and the number of samples

TABLE III

*Human excretion of nine metals (in μg./L. of urine)**

	Zn	Cd	Mn	Pb	V	Mo	Ni	Ag	Sn
Part I. Single 24 hour specimens from 24 normal Americans									
Mean of 24 specimens	318	<12.7	<26.7	120	<6.12	40.8	20.1	1.61	16.6
Standard error of the mean	31.9	1.20	2.91	35.5	1.55	4.32	2.59	0.15	1.84
Maximum	740	22	66	760	22	150	70	3.8	40
Minimum	82	<7.0	<12	5.0	<1.0	12	10	0.42	3.0
Number too dilute for accurate assay	0	7	6	0	10	0	0	0	0
Mean of 10 females	329	<10.9[5]	<23.4[3]	110	<3.98[4]	32.3	16.6	1.51	16.4
Mean of 14 males	310	<13.9[1]	<29.1[3]	126	<7.66[5]	46.9	22.6	1.67	16.8
Mean of 10 Negroes	335	<15.3[2]	<30.3[3]	149	<6.85[7]	44.7	23.4	1.50	17.9
Mean of 14 non-Negroes	307	<11.1[4]	<25.1[2]	97.2	<5.75[5]	29.0	18.1	1.67	15.8
Mean of 2 boys of 4 and 6 years	333	9.75	24.5	43.3	3.05	33.5	17.0	1.18	7.70
Mean of 2 men of 64 and 69 years	233	19.25	21.8	97.0	16.8	39.5	20.0	0.820	11.75
Part II. Twenty-four 24 hour specimens from one normal white American male of 24 years									
Mean of 12 "ordinary" specimens	279	12.9	20.8	91.1	<4.73	34.7	12.7	1.45	16.3
Standard error of the mean	16.2	1.10	1.77	11.4	1.19	6.62	1.24	0.46	1.42
Maximum	370	20	45	160	15	110	22	2.2	21
Minimum	170	7.3	13	48	<1.0	7.2	6.6	0.78	8.8
Number too dilute for accurate assay	0	0	0	0	3	0	0	0	0
Mean of 2 days with pH of 4.5	300	13.3	21.5	21.9	7.2	8.10	10.0	0.93	9.50
Mean of 2 days with pH of 7.8	215	18.5	19.9	43.5	11.9	21.5	11.0	1.05	35.0
Mean of 2 days with volume of 1,400 ml.	350	16.9	19.8	143	10.0	25.0	11.0	1.35	17.8
Mean of 2 days with volume of 8,425 ml.	305	13.2	21.0	75.0	1.58	10.0	11.6	1.83	7.3
Part III. Single specimens from 12 hospitalized African patients									
Mean of 12 hospitalized patients	798	<18.8	<88.4	38.9	<2.42	77.5	180	0.828	<10.7
Standard error of the mean	113	0.94	3.63	26.2	0.25	24.6	21.2	0.17	1.02
Maximum	1,400	33	560	220	8.8	200	500	1.8	25
Minimum	70	<14	<30	12	<1.6	12	27	0.48	<6.0
Number too dilute for accurate assay	0	1	7	0	8	0	0	0	4

* In order to define the metal excretion as completely as possible, the mean concentration with its standard error and the range of concentration with the number of specimens too dilute for accurate assay are tabulated in each of the three parts of the table. A < preceding a mean value indicates that at least one of the individual values was too small to determine accurately. For instance, the mean cadmium excretion is given as < 12.7 μg. per L. for the 24 normal Americans. The fifth row of figures indicates that seven of the samples contained less than the minimum accurately measurable amount, approximately 7.0 μg. per L. In calculating the mean of 12.7 μg. all 24 samples were considered, and the maximum value of 7.0 μg. was assigned to the seven cadmium-poor urines. Obviously the resultant mean is also a maximum value; however, it is smaller and therefore closer to the actual value than the mean of the 17 urines with measurable cadmium. For the female, male, Negro and non-Negro rows in Part I a superscript following a mean value indicates the number of samples too dilute to assay which went into calculation of that particular mean. Thus five of the ten female subjects had less urinary cadmium than could be accurately determined. Except for the Africans such uncertain means are confined to cadmium, manganese and vanadium.

in which a metal was too dilute to assay are also given. The average daily urine volume was 1,410 ml. Part I further compares the metal excretion of females and males, of Negroes and non-Negroes, and of children and old men. The tabulated data suggest no marked sex or race differences. Although not indicated in the table, none of the four white females excreted a measurable amount of vanadium. The urines of the patients in their teens and their fifties did not confirm the suggestive differences between the urines of the boys and their sexagenarian grandfathers with respect to cadmium, vanadium and lead excretion. The urines of the two pregnant women were not remarkable.

Part II of Table III lists the mean concentrations of the same metals in 12 "ordinary" 24 hour specimens from one normal adult. The standard error of the mean, the range of concentrations, and the number of samples in which a metal was too dilute to assay are also tabulated. The average daily urine volume was 1,590 ml. No change was evident in excretion pattern over the two years during which collections were made. Part II further compares the metal excretion in acidic and basic urine and in concentrated and dilute urine. On the four days during which ammonium chloride was ingested, urinary pH's were 5.7, 5.2, 4.6 and 4.4, respectively. On the four days during which sodium bicarbonate was ingested, urinary pH's

were 7.1, 7.3, 7.9 and 7.7, respectively. Because the effect of the maximum pH change was sought, only the final two days of each period were included in the table. The urine volumes for these final two day periods were 2,730 and 2,640 ml. for the acidic and basic urines, respectively. The 24 hour volumes of the two concentrated urines were 675 and 725 ml., their specific gravities were 1.027 and 1.028, and their pH's were 6.0 and 6.1. The 24 hour volumes of the two dilute urines were 4,350 and 4,075 ml., their specific gravities were 1.002 and 1.003, and their pH's were 5.8 and 6.2. Changing the urinary pH or concentration had no evident effect on zinc, cadmium, manganese, nickel or silver excretion which usually differed by no more than 30 per cent from the mean of the same individual's 12 "ordinary" specimens. Possible changes were confined to molybdenum, lead, vanadium and tin. The molybdenum content was between 20 and 50 μg. per L. of urine in 10 of the 12 routine specimens and in all the basic and concentrated specimens, whereas in the four acidic and the two dilute specimens it was between 7 and 11 μg. per L. There was less lead in each one of the four acidic urines than in any of the other specimens. Similarly there was more tin in each of the four basic urines than in any of the other specimens. The very large range of vanadium concentrations in the 12 "ordinary" urines made it impossible to evaluate the sixfold difference between the concentrated and dilute urines.

Part III of Table III lists the mean concentrations of the same metals in the urines of 12 hospitalized African Negroes. The standard error of the mean, the range of concentrations, and the number of samples in which a metal was too dilute to assay are also tabulated. Comparing single specimens from sick Africans and 24 hour collections from healthy Americans is difficult. In addition the smaller aliquots of African urine decreased the sensitivity of the method. The ranges of silver, tin, vanadium, lead, molybdenum, and cadmium concentrations were not obviously dissimilar for the Africans and the Americans, despite differences in their mean concentrations. In

TABLE IV

*Mean urinary excretion of nine metals for eight patients before, during and after intravenous $Na_2CaEDTA$ (in μg./L. of urine)**

Day	EDTA	No. pts.	Zn	Cd	Mn	Pb	V	Mo	Ni	Ag	Sn
Part I.	*Before $Na_2CaEDTA$ administration*										
1	0	5	221	$<13.3^1$	$<12.0^3$	208	11.4	19.0	19.1	1.02	22.4
2	0	6	298	$<12.0^1$	$<17.0^3$	118	12.3	21.4	23.5	1.19	21.4
3	0	8	259	$<14.4^2$	$<17.4^4$	210	9.9	20.4	15.0	1.12	19.0
4	0	8	264	$<12.1^3$	$<14.9^3$	89	7.1	23.1	17.0	0.95	19.6
		Mean	261	<13.0	<15.3	156	10.2	21.0	18.7	1.07	20.6
Part II.	*During $Na_2CaEDTA$ administration*										
1	1 Gm.	8	3,510	11.1	22.5	535	14.3	19.3	16.6	0.91	15.9
2	3 Gm.	8	2,840	14.0	39.0	483	13.5	16.8	15.5	0.85	15.2
3	3 Gm.	8	2,240	29.1	33.4	318	15.5	24.0	22.4	0.99	15.2
4	3 Gm.	7	3,250	26.2	31.5	625	15.4	19.4	24.8	0.94	20.3
5	3 Gm.	6	2,930	23.7	25.2	355	20.9	23.3	14.2	0.91	10.4
6	3 Gm.	5	2,930	29.8	24.9	253	22.6	24.0	17.9	1.13	12.8
7	3 Gm.	3	3,770	26.2	22.5	170	15.5	14.2	20.3	0.72	18.2
8	3 Gm.	3	2,550	28.8	41.0	245	20.3	21.5	16.8	0.98	17.8
9	3 Gm.	1	2,800	24.5	23.5	300	22.0	21.5	14.9	0.92	11.0
10	3 Gm.	1	2,100	26.0	20.5	120	20.0	19.0	17.0	0.82	11.0
		Mean	2,860	23.9	28.4	340	18.0	20.3	19.0	0.92	14.8
Part III.	*After $Na_2CaEDTA$ administration*										
1	0	4	2,490	24.5	20.6	270	4.96	16.1	13.8	0.80	21.6
2	0	3	567	$<14.8^1$	23.5	34	2.60	10.4	14.6	1.09	23.2

* Urine was collected on every day that $Na_2CaEDTA$ was given and the results are tabulated for all collections. The fact that there was only one patient for the ninth and tenth day, for instance, indicates that only one patient received $Na_2CaEDTA$ that long. The nephrotic patient who has already been reported (3) received 3 Gm. of $Na_2CaEDTA$ on the first two days, 4 Gm. on the third day, and nothing thereafter. Each of the mean values for cadmium and manganese concentration before $Na_2CaEDTA$ administration is a maximum value as indicated by the preceding <. The superscript indicates the number of samples too dilute to assay. In obtaining the means such dilute samples were assigned their maximum values which approximated 7 μg. per L. for cadmium and 12 μg. per L. for manganese.

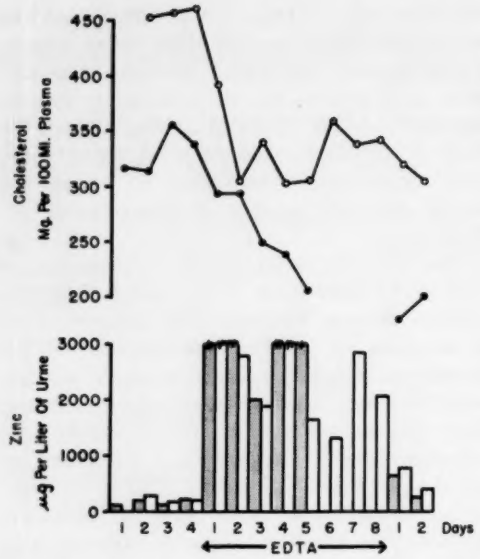

FIG. 2. THE INCREASE IN ZINCURIA AND CONCURRENT DECREASE IN PLASMA CHOLESTEROL FOR TWO ATHEROSCLEROTIC WOMEN

Daily cholesterol and zinc assays were made for both patients before, during and after administration of $Na_2CaEDTA$. The bar graph indicates the renal excretion of zinc, and the line graph indicates the level of plasma cholesterol. Stippled bars and solid dots represent one patient, while open bars and hollow dots represent the other. Four pre-$Na_2CaEDTA$ values are given for the former and three for the latter. The former received $Na_2CaEDTA$ for only five days, whereas the latter received it for eight days. Therefore the first post-$Na_2CaEDTA$ day for the patient receiving the shorter course was the day following the fifth day of chelate administration; the apparent break in her cholesterol curve does not indicate a three day period without assays but merely permits the post-$Na_2CaEDTA$ values of both patients to be plotted on the same graph. Similarly no urinary zinc levels are omitted. Since the maximum value of the standard working curve for zinc was 1,870 μg. per L., higher excretions required extrapolation. Excretions above 3,000 μg. were not even estimated for this graph.

contrast three of the Africans excreted significantly more zinc and another three excreted more manganese than any of the Americans. The most striking finding, however, involved nickel: Two-thirds of the Africans had urinary concentrations above the maximum American level and there was almost a tenfold difference between the mean concentrations of the two groups.

Table IV indicates the urinary excretion of metals before, during and after the administration of parenteral $Na_2CaEDTA$ to eight hypercholesterolemic patients. The patient with lead poisoning excreted more than 2,000 μg. of lead per L. of urine even during the control period; hence his lead concentrations have been omitted from the calculations. The most striking change during the administration of $Na_2CaEDTA$ was the prompt tenfold increase in zinc excretion. A smaller effect on cadmium and manganese excretion may well have been present but partially obscured by some control concentrations which were too low to quantitate. Suggestive increases also occurred in lead and vanadium excretion. The increase in cadmium, and perhaps vanadium, seemed to be somewhat delayed. Molybdenum, nickel, silver and tin excretions were apparently not affected. Moreover, no marked change in titanium or chromium excretion was noted. With the possible exception of manganese, the concentrations of urinary metals were returning to their pretreatment range within 24 hours of the time $Na_2CaEDTA$ administration was stopped. During the control period an average of 1,470 ml. of urine was excreted daily in comparison with 1,750 ml. of urine when $Na_2CaEDTA$ was administered dissolved in 500 ml. of 5 per cent glucose in water. Three of the eight patients had some decrease in their mean urine volume while receiving the chelating agent. No significant changes in urinary pH were observed during $Na_2CaEDTA$ administration.

Following intravenous $Na_2CaEDTA$, the level of plasma cholesterol fell in all eight patients, the average decrease being 96 mg. per 100 ml. plasma and the range being from 37 to 154 mg. The changes in plasma cholesterol and the concentrations of urinary metals, including iron, copper, titanium and chromium, have previously been presented as a case report for the nephrotic patient (3). In Figure 2 plasma cholesterol and urinary zinc are plotted on the same time axis for an additional pair of the eight patients, both of whom were atherosclerotic. A fourth patient, the one with pseudobulbar palsy, had cholesterol levels of 256, 273 and 267 mg. per 100 ml. plasma before he received $Na_2CaEDTA$. During the first seven days of his 10 day course of $Na_2CaEDTA$,

the daily cholesterol levels averaged 193 mg. per 100 ml. plasma with a minimum of 129 mg. Unfortunately no subsequent values were available. Ultracentrifugal studies on this patient's plasma, which were kindly performed by Dr. Robert H. Furman of the Oklahoma Medical Research Foundation, failed to reveal any specific differential effects on the various lipoprotein and Goffman fractions.

COMMENTS

Our inability to quantify chromium may have been due in part to loss of volatile chromyl chloride during digestion (4). The cause for the unsatisfactory titanium assay is not obvious. Although graphite is frequently contaminated by vanadium, it seems unlikely that such contamination played a major role in the relatively great variability among the 10 replicate vanadium analyses, since the metal was not found in the artificial urine to which it had not been added and since its standard working curve was linear.

The very real problem of contamination has been thoroughly reviewed (5). Wet ashing is the most dubious step. The required reagents are liable to introduce positive contamination; nitric acid is particularly difficult to free from metal impurities. Failure to detect transition or related metals when none was intentionally added to the artifical urine indicated that there was no serious positive contamination. Glass may adsorb metals which can later be eluted; etched beakers were avoided in an effort to minimize adsorbtion. The relatively constant analytic results for a single artificial urine with added metal and the essentially linear standard working curves suggested that negative contamination was not a major problem. With dissimilar urines accuracy would presumably be somewhat less.

As used here 8-hydroxyquinoline has been found to precipitate titanium, molybdenum, iron, cobalt, nickel and zinc quantitatively; thionalide precipitates any remaining manganese, copper, silver, cadmium, tin and lead; and tannic acid completes the precipitation of vanadium and chromium (2). Presumably the combination of these three metal-binding agents would precipitate all the elements in the fourth period from titanium (atomic number 22) to arsenic (atomic number 33). Moreover, the corresponding elements of the fifth and sixth periods would probably be precipitated similarly.

The urine of normal American adults contained measurable amounts of transition and related elements. Excretion of iron, copper, zinc, molybdenum, manganese and cobalt was expected since they are essential trace elements. All but cobalt were found, and presumably its apparent absence was due to insensitivity of our method since Harp and Scoular have reported that the daily renal excretion approximated 5 μg. (6), an amount which is well below one-twentieth of what we added as an internal standard. On the other hand, titanium, vanadium, chromium, nickel, silver, cadmium, tin and lead have no known biologic function, although each was found in every urine specimen. Tipton and co-workers observed measurable quantities of all these metals, except vanadium, in many adult human tissues from this country (7); moreover the other nonessential transition metals which they did not find in tissues (7) were not observed in the urine.

The age and geographic origin of human beings affect not only the concentrations of cadmium, in their kidneys (8) but also of other metals in many organs (9). The urine of non-Westernized African natives was examined because such people presumably had less exposure to metals. The source and the significance of their excreted nickel is not evident; no obvious means of contamination with this metal was found.

For most of the metals considered here, few excretion studies in man are available. Published data on urinary zinc approximate our findings. In the urine of normal individuals McCance and Widdowson found an average of 0.3 mg. of zinc per day; they also found that neither oral nor parenteral administration of zinc increased its renal excretion, although proteinuria was accompanied by a sevenfold increase in zincuria (10). Vallee, Wacker, Bartholomay and Hoch found a mean normal excretion of 440 μg. of zinc per L. of urine with a standard deviation of 210 μg.; in contrast for patients with postalcoholic cirrhois of the liver these authors found an excretion of 850 μg. of zinc per L. of urine (11). For normal individuals Kehoe, Cholak and Story found 27 μg. of lead, 11 μg. of tin, less than 10

μg. of manganese, and less than 5 μg. of silver per L. of urine (12). Smith and Kench found that 10 subjects with no known exposure to cadmium excreted from 2 to 22 μg. of that metal per L. of urine (13).

The level of cholesterol in human plasma falls following parenteral $Na_2CaEDTA$ (1). Since this compound is not metabolized *in vivo* (14), its effect is presumably related in some way to its ability to chelate metals. Except for cobalt, the renal excretion of the entire first transition series of elements from titanium through copper has been investigated following the administration of $Na_2CaEDTA$. Urinary concentrations of titanium and chromium were only roughly approximated. The concentrations of iron and copper were previously reported for one of the patients (3). $Na_2CaEDTA$ apparently increased the excretion of manganese, vanadium and iron; however, the most striking effect was the increased excretion of zinc which is not a transition metal. Cadmium and lead, which are also not transition metals, were somewhat affected.

There was no excessive zincuria in our only patient with proteinuria (3); however, improvements in the assay technique have required revisions in the previously published nickel and lead excretions before, during and after $Na_2CaEDTA$. The tabulated nickel concentrations must be revised slightly upward and the lead concentrations must be multiplied by approximately 100. The very low cadmium concentrations which were reported in the same patient were obtained by using the 2,288.0 Å cadmium line; his delayed increase in cadmium excretion following $Na_2CaEDTA$ is striking (3).

SUMMARY

A method of simultaneously assaying nine trace metals in human urine has been described and the following data obtained by its use:

1. The mean urinary concentrations of zinc, cadmium, manganese, lead, vanadium, molybdenum, nickel, silver and tin were determined for 24 normal adults. Standard errors of the mean and ranges of concentration have been tabulated.

2. Day-to-day variations in the renal excretion of these metals and the changes occurring in acidic, basic, dilute and concentrated urine were measured for one normal adult.

3. The urinary metal was compared for healthy Americans and hospitalized Africans. The most suggestive difference was the greater nickel excretion by the latter.

4. The changes in metal excretion during the administration of disodium calcium ethylenediaminetetraacetate to hypercholesterolemic patients were followed. Marked increases in zinc were universal and lesser increases were observed in cadmium, manganese, lead and vanadium. A fall in plasma cholesterol occurred simultaneously with the increases in urinary metal concentrations.

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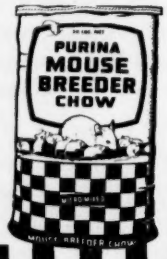

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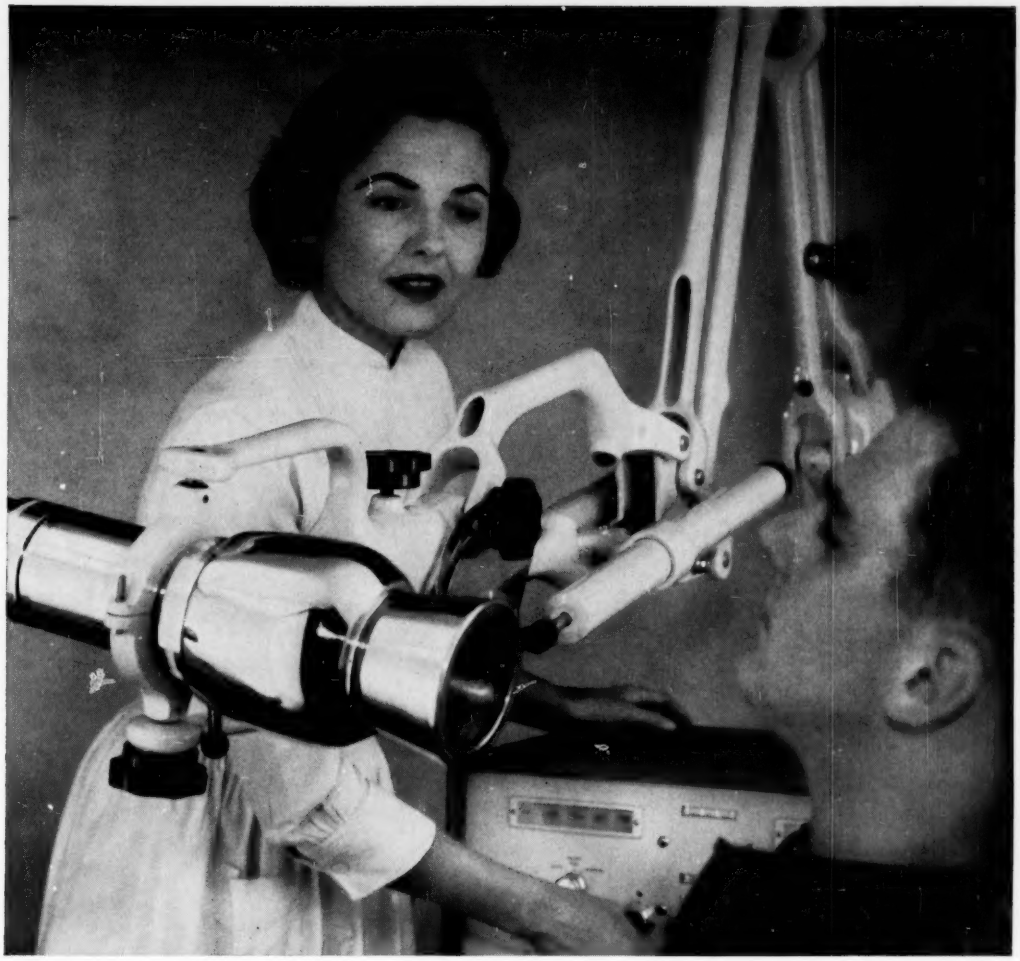

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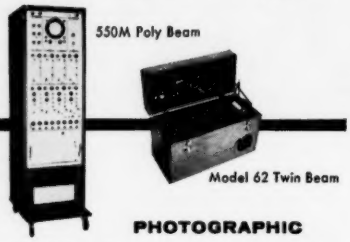

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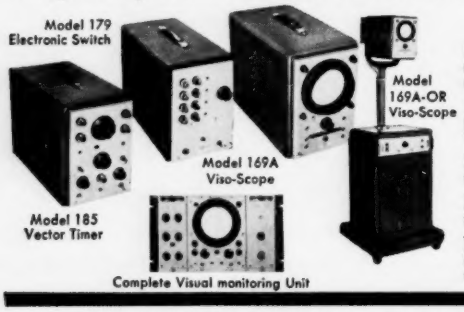

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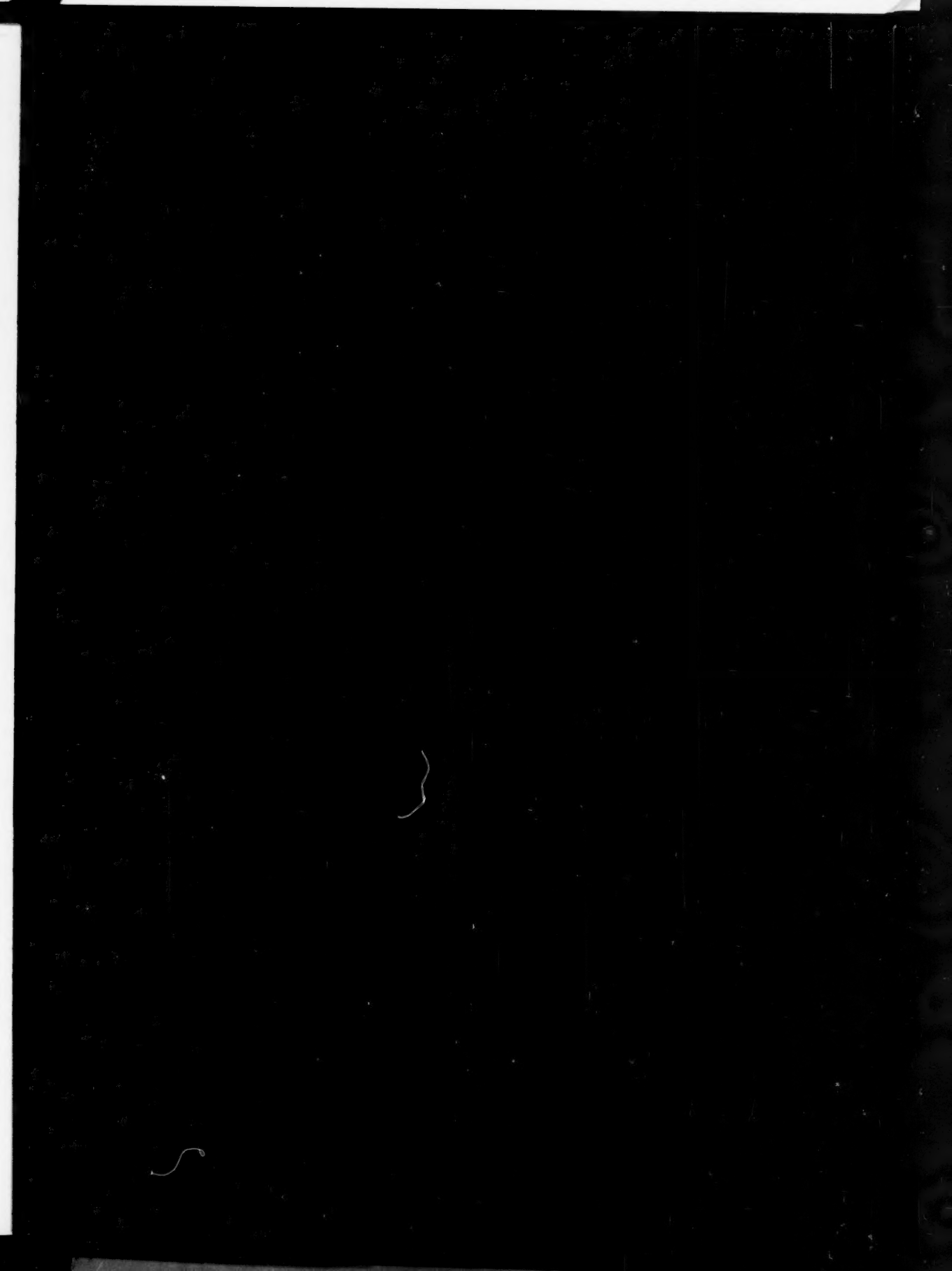
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